THE
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THE
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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

A QUANTITATIVE STUDY OF PHENOCOPY PRODUCTION WITH MONOCHROMATIC ULTRAVIOLET IRRADIATION

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The term phenocopy was introduced by Goldschmidt (1935) to refer to forms, produced by some experimental procedure, whose appearance duplicates or copies the phenotype of some mutant or combination of mutants. The first experiments with phenocopy production were those of Standfuss (1896) who, by treating butterfly pupae with high or low temperatures, produced adults which resembled other geographic races. Phenocopies have been produced in Drosophila by high temperature (Goldschmidt, 1929, 1935; Plough and Ives, 1932, 1935; Child, Blanc, and Plough, 1940), low temperature (Gottschewski, 1934), X-rays (Friesen, 1936; Waddington, 1942; Villee, 1946a), chemical agents (Rapoport, 1939), visible light (Villee and Lavin, 1946), and ultraviolet light (Geigy, 1931; Eloff, 1939; Epsteins, 1939). Geigy irradiated only very early egg stages and obtained flies with abnormal abdomens, legs, and wings. Eloff was interested primarily in the effects of ultraviolet light on crossing-over but observed some wing abnormalities when late pupae were irradiated. Epsteins irradiated larvae and pupae and produced abnormal abdomen, hemithorax, the absence of the scutellum, and abnormalities in the wings, chiefly scalloping of the distal and posterior edges of the wings. In none of these experiments was the intensity of the ultraviolet light measured.

This study was undertaken to provide quantitative data of the effects of ultraviolet light on larval and pupal stages. It was originally planned to use both 2537 A and 2800 A light to see if the phenocopy-producing reactions could be ascribed to changes in nucleic acid or protein metabolism. However, a light source providing 2800 A light could not be obtained so experiments with that have been postponed. Davis (1944) made a quantitative study of the effects of ultraviolet in inhibiting the folding process in neural tube formation in chicks. By using monochromatic light of different wave-lengths obtained from a monochromator he determined the photochemical efficiency curve for the process. This was found to compare closely with the absorption curve of sterols, especially that of 7-dehydro-

[1] I am greatly indebted to Dr. George I. Lavin for supplying the ultraviolet lamp, filter, and meter used in these experiments and for furnishing much invaluable advice and criticism during the course of the study, and to Dr. Eric G. Ball for the facilities of his laboratory.

cholesterol, with maxima at 2576 A and 2804 A. Schechtman (1944) found that the inhibiting effects of ultraviolet light on the development of Hyla eggs were slightly stronger at 2537 A than at other bands tested and Landen and Uber (1939) found that the inactivation of yeast by ultraviolet was greatest at 2600 A, where 500 ergs/mm.^{2} produced 50 per cent inactivation. Stadler and Uber (1942) found that the photochemical efficiency curve of ultraviolet light in producing mutations in maize corresponded to the absorption curve of nucleic acids and Hollaender (1945) and colleagues found that ultraviolet of wave-length 2600 A was much more efficient than other wave-lengths in producing mutations in a variety of fungi, Neurospora, Trichophyton, Penicillium, and Aspergillus.

Materials and Methods

Three stocks were irradiated: a wild type and an aristapedia-Bridges (ss^{aB}, chromosome 3, locus 58.8) stock isogenic with it (Villee, 1946b) and an independent, miniature wing (m, chromosome 1, locus 36.1) stock. Larvae were obtained by allowing large numbers of stock flies to lay eggs for a two-hour period on corn meal-molasses-agar food in half-pint bottles. The ages of the larvae are thus known to within $\pm$ one hour. The cultures were kept at 25.5° C. before and after irradiation. At this temperature the larvae pupate within a few hours of 100 hours after they hatch from the eggs. Prepupae (white pupae) were collected frequently and the time noted. The age of the pupae at irradiation was determined from this. A total of 3,500 flies was irradiated in groups of 25 larvae or pupae.

The source of the ultraviolet radiation was a spiral quartz mercury resonance lamp, manufactured by the Hanovia Chemical Company, which emits about 80 per cent of its energy in the form of the 2537 A line. A 120 mA. luminous tube transformer was used. The visible radiation was removed by a quartz filter cell containing a mixture of nickel sulfate and cobalt sulfate dissolved in distilled water (Backström, 1940). The lamp used had been burned well over 100 hours before the experiments began so the amount of radiation of wave-lengths shorter than 2537 A should be negligible. The intensity of the irradiation was varied by altering the distance between the lamp and the target. The intensity was measured by a Hanovia ultraviolet meter, the target of which was fastened to a carriage on an optical bench. The larvae or pupae to be irradiated were placed in a small uncovered Petri dish on this carriage. By moving the carriage back and forth, intensity measurements were taken before and after each irradiation and always checked very closely.

The larvae to be irradiated were removed from the culture bottles, washed briefly in 70 per cent alcohol, rinsed in Ringer's solution, dried on filter paper and placed in small, uncovered, dry Petri dishes. The larvae very shortly became stuck to the glass and showed no tendency to escape from the dish. After irradiation, the larvae were moistened with Ringer's solution to free them and removed with a camel's hair brush to shell vials containing culture medium to complete development. This drying treatment had no deleterious effect on the larvae: several groups of larvae were handled in this way and dried 20 to 30 minutes without irradiation and all hatched out normally.

Results

Larvae show a gradual increase in sensitivity to ultraviolet radiation with age from 50 to 100 hours after hatching to a maximum at one hour after pupation, then a sharp decrease in sensitivity with pupal age. The sensitivity of flies to ultraviolet at an intensity of 44 ergs per mm.^{2} per second for different durations of exposure, as measured by the percentages killed, is given in Figure 1.

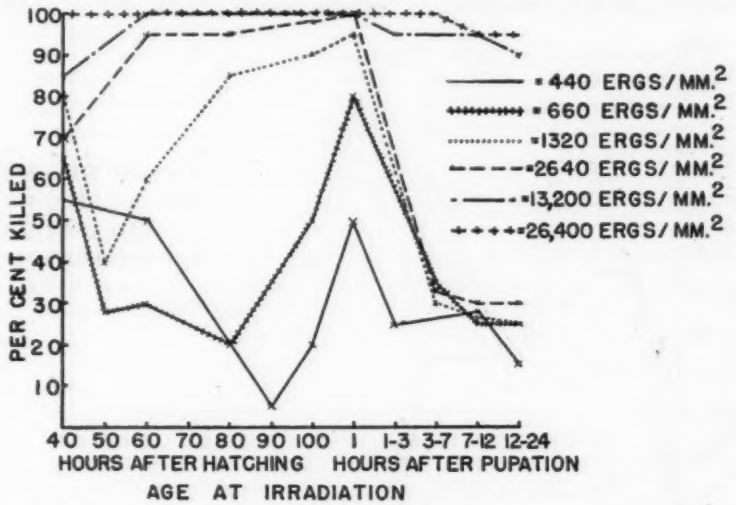

Figure 1. Percentage of flies killed by different total dosages of irradiation at different ages given at 44 ergs/mm.^{2}/sec.

The percentage of flies showing phenocopies also varies with the age of the larva or pupa irradiated (Fig. 2). There seem to be two periods during the larval stage of greater phenocopy production for a given amount of radiation, one about 50 and one about 100 hours after the larvae hatch from the egg. The entire larval period from 40 to 100 hours after hatching is one of fairly high phenocopy production, higher than the first 24 hours of pupal life. Some pupae showed very high percentages of phenocopies, up to 400 per cent (400 phenocopies per 100 flies), but this was caused by the fact that the pupae can withstand more energy for the production of phenocopies without being killed. The high percentages of phenocopies were the result of irradiations with 13,200 or 26,400 ergs/mm.^{2} Since these dosages killed 100 per cent of the flies irradiated at most of the ages used, no lines were drawn for them on Figure 2. In 7- to 12-hour pupae, irradiations of 3,960 ergs/mm.^{2} gave 120 phenocopies per 100 flies, 13,200 ergs/mm.^{2}, 200 phenocopies per 100 flies, and 26,400 ergs/mm.^{2}, 300 phenocopies per 100 flies.

The phenocopies produced included abnormal abdomen, combgap legs, abnormal thorax, small or rough eye, fused eye facets, folded, dumpy, curled or balloon wings, abnormalities in the wing veins, fused, singed or missing bristles and microchaetes,

doubled sex combs on a male prothoracic leg, and a shoulder-like protrusion growing anteriorly from the mesothorax.

The most numerous type of phenocopy produced was that involving an abnormality in the abdomen, some deformity in or the complete absence of one or more tergites. These were produced by irradiating flies in any stage of development from 40-hour larvae to 24-hour pupae. Geigy (1931) produced similar phenocopies by irradiating eggs ½ to 17½ hours old. (See Geigy for figures of the variations in phenotype produced.) Epsteins (1939) reported similar abnormali-

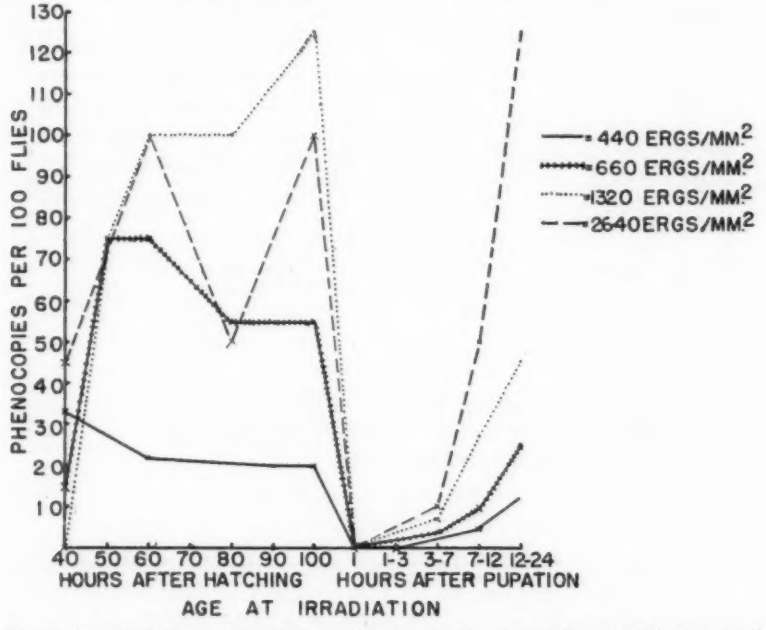

FIGURE 2. Number of phenocopies per 100 flies produced by different total dosages of irradiation at different ages given at 44 ergs/mm.²/sec.

ties produced by irradiations at times comparable to those used in this study. The large numbers of these resulting from irradiations during either the egg, larval, or early pupal stage is probably due to the fact that the dorsal abdominal imaginal discs lie close to the dorsal surface and are more readily reached by the rays than are deeper-lying discs. Eloff (1939) showed that ultraviolet light penetrates one thickness (about 10 microns) of Drosophila cuticle with only slight diminution of effect, but that four thicknesses screen out the effective rays.

Abnormalities in the formation of the mesothorax and scutellum occur after irradiation during the pupal period, especially between 7 to 12 and 12 to 24 hours after pupation. These include absence of the scutellum, failure of the right and left dorsal mesothoracic discs to fuse, failure of one disc to unfold properly, etc., giving phenotypes similar to those of certain grades of tetraltera (see Villee, 1942,

for figures). The eye phenocopies, small, rough, or fused eye facets, also were obtained after irradiation in the pupal period, especially between 7 and 24 hours after pupation. One fly irradiated in this period showed large, swollen legs resembling the phenotype of combgap. The wing and bristle phenocopies were produced primarily by irradiation in the pupal period, 7–24 hours after pupation. Reduction of the microchaetes occurred only after irradiation in this period. An additional period in which both bristle and wing phenocopies were produced was between 80 and 90 hours of larval life. Dumpy wings were produced only by the irradiation of the pupae.

The three stocks used in general responded similarly to irradiations of comparable developmental stages. The single exception was aristapedia-Bridges, which frequently developed 2- to 4-segmented tarsi when irradiated as 80- or 90-hour larvae or as 7- to 12-hour pupae. This same stock also develops very short, 1- to 4-segmented tarsi when treated with X-rays (Villee, 1946a), low temperature (Villee, 1943) or when combined with "growth rate" genes (Villee, 1945), although under normal conditions it always has normal 5-segmented tarsi (see Villee, 1946a for discussion).

Only two phenocopies were found which resembled the pervasive effects of overgrowth or abnormal histogenesis found frequently with X-radiation of 70- to 90-hour larvae (Waddington, 1942; Villee, 1946a). One wild type male irradiated as a 3- to 7-hour pupa with a total of 5,280 ergs/mm.^{2} developed a second pair of sex combs on the second tarsal segment of the prothoracic leg, and resembled closely a phenotype obtained with X-radiation (Villee, 1946a, Fig. 8). One wild type female irradiated 100 hours after hatching from the egg, about 2 hours before pupation, with 2,640 ergs/mm.^{2} developed a small palp-like outgrowth from the anterior dorso-lateral margin of the thorax, apparently identical with those obtained with X-radiation (Villee, 1946a, Figs. 1–3).

Irradiation of larvae with ultraviolet does not cause a retardation of pupation as X-radiation does. The larvae pupate in the normal time after ultraviolet treatment; X-radiation causes a retardation of pupation of 8 to 12 days beyond the time when controls pupate (Villee, 1946). With X-radiation there is also a marked difference in the results of irradiation of about the same dosage given at different intensities. A given dosage at high intensity causes a much higher lethality than a similar dosage at low intensity. With ultraviolet radiation, this difference is not found. A given dosage of ultraviolet light, whether given at 44, 37, 27, 17.5, or 11 ergs/mm.^{2}/sec., produces about the same percentage of lethality and of phenocopies. It may be that the range of ultraviolet intensities used was not great enough. There is only a factor of four between the lowest and highest intensities, whereas in the X-ray experiments the high intensity was 71 times the low intensity (5,540 *vs.* 78 r. units per minute). However, Carlson and Hollaender (1944) found that the effects of 2537 A light on mitosis in grasshopper neuroblasts depend simply on the total dosage and not on the intensity even when it is varied by a factor of 1500. In a later paper (Carlson and Hollaender, 1945) they found that at low total dosages (57.6 ergs/mm.^{2}) intensities varying from 0.004 to 16.3 ergs/mm.^{2}/sec. showed no significant differences in the effect on mitosis, but at high total doses (172.8 and 230.4 ergs/mm.^{2}) treatments given at high intensity were slightly more effective in depressing mitosis than ones at low intensity. The dosages used in this study were higher than Carlson and Hollaender's highest but

no intensity effect was observed. Bain and Rusch (1943) reported just the opposite conditions in the production of tumors in mice by ultraviolet radiation. They found ultraviolet of wave-lengths 2800–3400 A more effective in producing tumors when given at low intensities over long periods of time than when given at high intensities for short periods. They used much greater amounts of energy, 116–212 $\times 10^5$ ergs/mm.^{2}, than used by Carlson and Hollaender in their experiments. The high intensity, 1.35 $\times 10^5$ ergs/mm.^{2}/day, was about four times their low intensity, 0.35 $\times 10^5$ ergs/mm.^{2}/day.

Discussion

The factors regulating the production of phenocopies by temperature treatments are: (1) the developmental stage at which the treatment is applied; (2) the extensity (total time) of the treatment; (3) the intensity of the treatment; and (4) the genotype of the animals treated (Goldschmidt, 1929, 1935). With ultraviolet light, the important factors are the age of the fly at irradiation, the stock used, and the total dosage of irradiation. Within the range of intensities used in these experiments, variations in intensity had no effect in changing the percentage of lethality or of phenocopies. The phenocopies produced may, in the main, be explained as due to the absorption of the light energy in individual cells or small groups of cells, probably by the nucleic acids, since 2537 A is close to the region in which they absorb maximally, 2600 A. Since both desoxyribose nucleic acid, located in the chromosomes, and ribose nucleic acid, located in the cytoplasm as well as in the chromosomes, absorb at the same wave-length, it is impossible to decide whether the phenocopy-producing reaction is localized in the nucleus, cytoplasm, or in both. The fact that ultraviolet of 2537 A wave-length affects the chromosomes and retards mitosis in grasshopper neuroblasts (Carlson and Hollaender, 1944) would suggest that the phenocopy effect is also mediated by the chromosomes. Hollaender, Greenstein, and Jenrette (1941) found that 2537 A radiation causes a depolymerization of sodium thymonucleate (desoxyribonucleate) *in vitro*. As a working hypothesis we may suppose that irradiation of Drosophila larvae or pupae with 2537 A is absorbed by nucleic acids or nucleoproteins in the chromosomes of the cells of the imaginal discs near the surface. The absorption of this energy results in a physical change, a depolymerization, of the nucleic acid with a consequent upset in the structure of the gene so that it is partially or completely inactivated, with the result that development of that structure is abnormal. Since the inactivated genes are located in some body cell rather than a germ cell, a phenocopy rather than a mutation is produced. Future research may, of course, show that the phenocopy-producing mechanism is entirely different from that proposed here.

Some of the phenocopies found in these experiments suggest that the cells of the imaginal discs were killed, others suggest that certain genes were altered or inactivated, perhaps by the scheme outlined above. Only two of the phenocopies, a doubling of the sex combs on the male prothoracic leg, and a palp-like outgrowth from the anterior dorso-lateral margin of the thorax, each of which occurred only once in the course of the study, suggest the pervasive effects of abnormal histogenesis found frequently with X-radiation. These have been explained (Waddington, 1942; Villee, 1946a) by assuming that the X-rays cause the death of cells

and that the dead or necrotic cells release diffusible morphogenetic substances which result in the abnormal histogenesis or overgrowth. The same explanation may be applied to the abnormal histogeneses produced by ultraviolet radiation.

The sensitive periods for certain of the phenocopies are slightly different from those found for X-rays or temperature treatments. It is rather difficult to compare the work of different investigators, who use different stocks raised at different temperatures, but it would appear that the sensitive period for the production of dumpy wings by ultraviolet corresponds with that found by Blanc and Child (1940) for temperature treatments and that the larval sensitive period for bristle reduction by ultraviolet is identical with the temperature sensitive period determined by Child (1935). In addition there is a sensitive period for bristle reduction by ultraviolet in the pupal stage which was also found in X-radiation experiments (Waddington, 1942; Villee, 1946a). The sensitive period for the reduction of the size of the eye by ultraviolet is in the pupal period, 7–24 hours after pupation, whereas Goldschmidt (1935) found the temperature sensitive period to be in the larval period, at an age corresponding to approximately 90 hours after hatching from the egg. Abnormalities in the thorax appear after ultraviolet irradiation in the pupal period, 7–24 hours after pupation, but in the larval period at ages corresponding to 50–100 hours after hatching from the egg following X-radiation (Villee, 1946a). The wing phenocopies produced by ultraviolet had two sensitive periods, one in the larval period about 80–90 hours after hatching from the egg, which corresponds to the temperature sensitive period (Goldschmidt, 1935) and an additional one in the pupal period not found with temperature treatments.

The energy threshold for the production of phenocopies is slightly below the lowest total dosages, 330 and 440 ergs/mm.^{2}, used in these experiments and varies with the age of the fly irradiated. At one hour after pupation, no phenocopies were produced by 440 ergs/mm.^{2} but the dosage caused a 50 per cent mortality among the pupae irradiated. At other ages, from 5 to 33 per cent phenocopies (i.e., 5 to 33 phenocopies per 100 flies) were obtained with this dosage. This threshold level is of the same order of magnitude as that found by Landen and Uber (1939) for the inactivation of yeast (500 ergs/mm.^{2} produced a 50 per cent inactivation) and by Giese (1946) for the production of abnormalities in developing echinoderms by the irradiation of sperm before fertilization. He found the threshold level for the production of abnormalities in development by irradiating the eggs of these forms to be considerably higher, on the order of 5,000–8,000 ergs/mm.^{2} Carlson and Hollaender (1945) found a considerably lower threshold in the effects of ultraviolet on mitosis in grasshopper neuroblasts: about 100 ergs/mm.^{2} produce a reduction of the mitotic ratio to 0.5. Davis (1944) found that the energy required to inhibit the folding process in neural tube formation varied with the wave-length of the ultraviolet used and that about 200 ergs/mm.^{2} produced the inhibition when ultraviolet of 2537 A was used.

It is impossible to make an exact comparison between the actions of X-rays and ultraviolet rays per energy unit, first, because their modes of action are different, and second, because only a small percentage of the X-rays are absorbed and become effective in the tissue irradiated, the rest pass through without affecting the cells, whereas ultraviolet rays are largely absorbed by tissues as thick as a Drosophila larva or pupa. However, it is of interest to make a rough comparison between the experiments reported here and the previous study using X-rays

(Villee, 1946a). It was found that the threshold for the production of phenocopies with X-rays was slightly below 1096 r. units. At that level from 0 to 40 per cent of the flies were killed and from 0 to 10 per cent showed phenocopies. This dosage D in roentgens may be converted to the density of absorbed energy, E, by the formula $E = 83\ D$ ergs/cm.^{3} (Cole, personal communication) to give 90,968 ergs/cm.^{3} The absorbed energy of the ultraviolet radiation, computed from the area and volume of the larva and the minimal threshold intensity of 440 ergs/mm.^{2} and assuming total absorbtion by the organism, is 440,000 ergs/mm.^{3} From this it can be seen that X-radiation is approximately five times as effective per energy unit as ultraviolet radiation of wave-length 2537 A in producing phenocopies. I want to thank Professor Kenneth S. Cole for his assistance in making these calculations.

Summary

1. Drosophila larvae, prepupae and pupae of various ages and genotypes were irradiated with ultraviolet of wave-length 2537 A and in dosages varying from 330 to 79,200 ergs/mm.^{2} The phenocopies produced varied with the age of the irradiated fly, the stock used, and the total dosages of the irradiation. Irradiation with ultraviolet does not cause a retardation of pupation as X-radiation does.

2. Larvae show a gradual increase in sensitivity to ultraviolet radiation with age from 50 to 100 hours after hatching from the egg to a maximum at one hour after pupation, then a sharp decrease in sensitivity with pupal age. There are two periods in the larval stage, one about 50 and one about 100 hours after hatching, of greater phenocopy production for a given amount of radiation. Irradiations during the first 24 hours of pupal life produce fewer phenocopies for a given amount of radiation than during the larval period.

3. The sensitive periods for the production of certain phenocopies by ultraviolet are compared with the sensitive periods for X-ray and temperature treatments. Some are identical, a few are different.

4. Irradiations of the same total dosage produce the same percentages of lethality and of phenocopies whether given at high or low intensities. The threshold level for the production of phenocopies varies with the age of the fly irradiated but is about 440 ergs/mm.^{2} A comparison is made of this threshold with the thresholds for the effect of ultraviolet on other biological systems and with the effect of X-rays on phenocopy production in Drosophila.

5. A hypothesis for the mechanism involved in the production of phenocopies by ultraviolet rays is discussed.

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CYTOLOGICAL AND EXPERIMENTAL STUDIES ON THE OÖCYTES OF FRESH WATER PULMONATES [1]

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The organization of the ovum and the importance and relation of its inclusions such as yolk, oil, pigment, and other granules, to the ontogenic process are of considerable interest to embryologists.

Early centrifugation experiments on the eggs of many invertebrates established the facts that distribution of the visible elements apparently has no direct causal relation to the cleavage pattern of the egg or the development of the adult, and that there must be some sort of formed or underlying ground substance which could be only slightly affected, if at all, by centrifugal force (Lillie, 1906, 1909; Morgan, 1908; Morgan and Spooner, 1909; Conklin, 1910). More recently Raven and Bretschneider (1942), as the result of experiments with the eggs of the pond snail, *Lymnaea stagnalis,* have questioned the premise that distribution of visible elements in the egg has no effect on the developmental pattern. However, they have been unable to cause any atypicalities in ontogeny attributable to centrifugation and stratification of these elements.

Practically all of the centrifugation experiments directed at gaining information about the organization of the ovum or the role of the visible inclusions in development have been made on eggs already deposited, and, in many cases, already fertilized. Few, if any, have considered the egg from the point of view of its development prior to its release from the gonad, during the time when its organization might be considered in the "formative stage."

The present paper is a report of some cytological and experimental investigation on the ovarian oöcytes of two species of fresh water snails, *Lymnaea stagnalis appressa* Say and *Physa gracilis.* These species were chosen for investigation because their eggs exhibit a spiral type of cleavage which is determinate. Hence, they may be said to be "restricted" at a very early stage in development. Moreover, the potentialities of the deposited eggs of numerous species in the genera *Physa* and *Lymnaea* have already been thoroughly investigated and hence there has accumulated much information against which new research can be contrasted and properly evaluated.

The cultures of *Physa* were obtained from laboratory stocks on hand at the Biological Laboratories of Harvard University, while those of *Lymnaea* were imported to the laboratories from Wisconsin. Both cultures were kept in laboratory aquaria where they reproduced readily without special culture techniques other than those usually followed in maintaining balanced aquaria.

[1] The experimental work described herein was done at the Biological Laboratories, Harvard University, Cambridge, Mass.

* The writer wishes to express his sincere gratitude to Professor Leigh Hoadley for his kindness, generosity, and many helpful suggestions pertinent to the course of this work.

The body of the work may be divided into three parts. The first includes a cytological comparison of the developing oöcytes of *Lymnaea* and *Physa*. The second deals with an analysis of the effects of centrifugation upon the ovarian oöcyte, and the third is concerned with a comparison of the effects of X-radiation on the two species.

For all cytological studies, unless otherwise indicated, Champy fixing fluid was used, followed by the Champy-Kull staining procedure. The gonads were excised and then fixed, since the size of the snails made it impractical to obtain good fixation by immersion of the entire animal. Material was embedded in paraffin and sectioned at five microns.

The Developing Oöcyte

As might be expected from the relative sizes of the two species, the gonad of *L. stagnalis* is considerably larger than that of *P. gracilis*. In both species, the hermaphroditic gland is found embedded in the liver on the inner side of the coiled visceral "hump." The organ is lobed and slightly more compact in its consistency than is the surrounding liver tissue. It may be found easily by tracing the prominent white and convoluted oviduct to the point where it appears to enter the body of the liver. This point marks the anterior aspect of the gonad.

The superficial histological picture of the active ovotestis presented by the two species is essentially the same. In both one finds numerous tubules in which the growth and maturation of sperms and the development of oöcytes goes on simultaneously. The oöcytes are situated along the walls of the tubules and may be identified quite early in development by their location and by their enlarged nuclei. At the earliest recognizable stage they measure about 10–15 μ in diameter, while at the time of release from the gland the diameter has increased to approximately 130–140 μ. In spite of the difference in the size of the adults of the two species, the size of the ova is about the same. For an account of the general histology of the mature ovotestis, the reader is referred to the work of Crabb (1927) in which the species *Lymnaea stagnalis* is treated at considerable length. The present report will present only sufficient descriptive background to clarify fully the position and relations of the specific regions of the gonad under consideration.

With Champy-Kull fixation and stain, the ovarian oöcytes of both *Lymnaea* and *Physa* show evidences of a clearly marked polar differentiation by the time they have reached one-third of their maximum size. The germinal vesicle is displaced slightly toward one pole of the cell and a center of accumulation of droplets or granules which are blackened by osmic acid is present in the region of the opposite pole. Yolky granules which stain red fill the cell completely and obscure details of any other cytoplasmic inclusions which might be present. The yolk granules show a slight gradation along the axis of polar differentiation, being a little finer in the region of the germinal vesicle and coarser at the opposite end of the cell (Plate *1A* and *B*; Fig. 1*A*). A detailed study of the morphological evidences of the polarity reveals certain differences in *Physa* and *Lymnaea* which are expressed through the movements of the osmiophilic inclusions during the development of the oöcyte.

In *Lymnaea*, these black droplets appear first when the egg is about 25 μ in diameter. They arise in the vegetative region and remain there until after the

release of the oöcyte. As the oöcyte increases in size, the number of the globules in the mass increases until the vegetal pole becomes literally packed with them (Fig. 1*B*). Following shedding of the oöcyte, the osmiophilic inclusions spread out from their massed apolar position and invade the region of the red-staining yolky granules, with the result that the visible polarity begins to be obscured

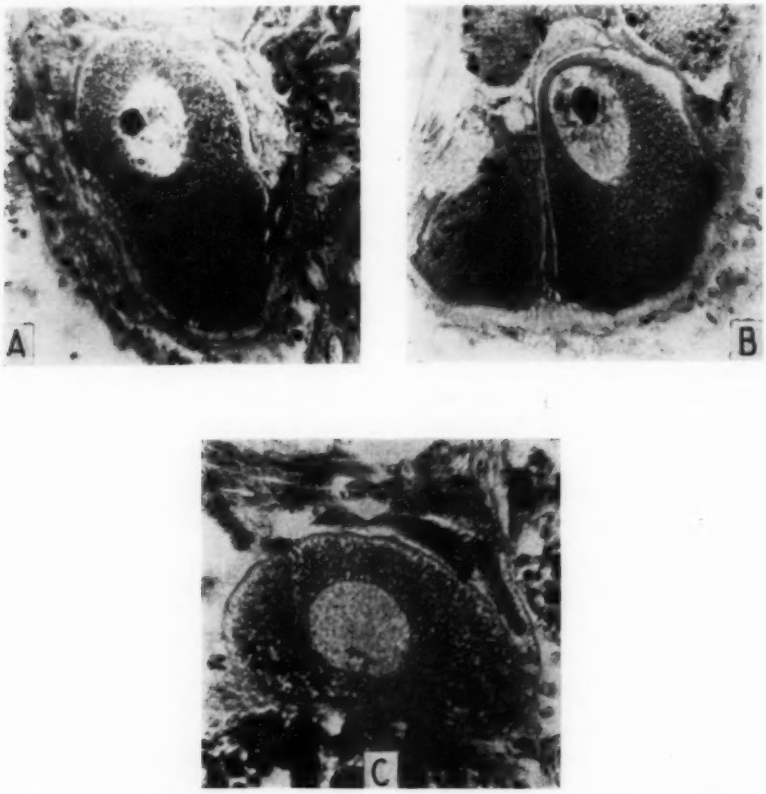

PLATE I. Photomicrographs of oöcytes within their envelopes in the hermaphroditic gland. *A* and *B*, *Lymnaea*; *C*, *Physa* (× approximately 500)

(Fig. 1*C*). This migration starts before any signs of breakdown appear within the germinal vesicle, and progresses more rapidly in the subcortical portion of the cell. With Champy-Kull stain it is difficult to determine whether this migration is the result of factors instigated solely within the oöcyte, or whether it is the result of an activation caused by fertilization. The work of Crabb (1927) on *Lymnaea* argues strongly for the latter explanation. But regardless of the cause of its origin, it is to be stressed that the pattern of migration is constant for all oöcytes, and the osmiophilic material always has the same distribution in oöcytes

at comparable stages of development. By the time the maturation process has begun and the nucleus is no longer intact or visible the osmiophilic mass appears equally distributed throughout the cell (Fig. 1*D*).

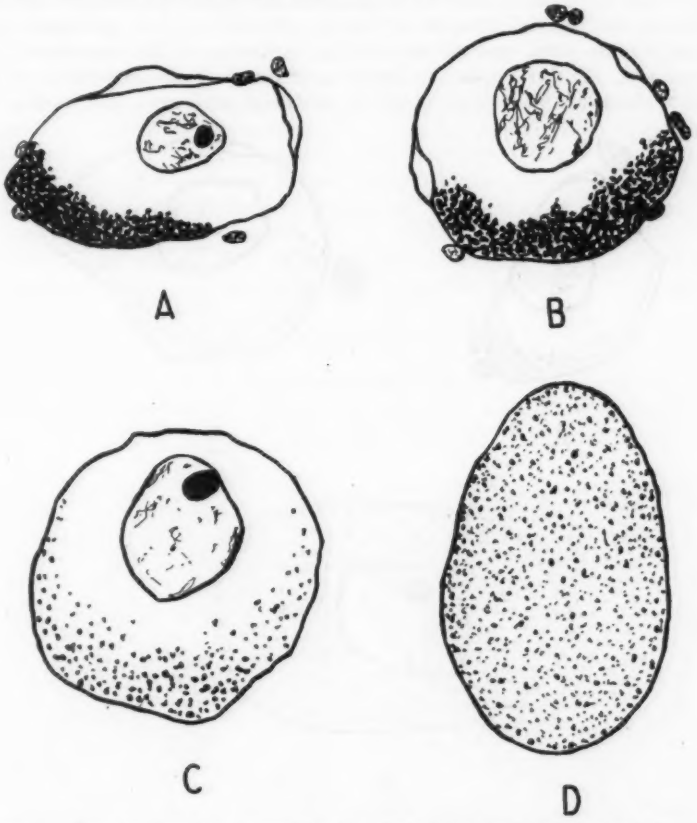

FIGURE 1. Sketches based on camera lucida tracings to show distribution of osmiophilic material during the course of development of the oöcyte of *Lymnaea*. *A* and *B*, ovarian oöcytes; *C*, oöcyte at the time of release from surrounding envelope; *D*, oöcyte in tubule of gonad just after breakdown of germinal vesicle.

In *Physa* the darkened globules appear first when the oöcyte is about 25–30 μ in diameter. Though there is some accumulation of these globules in the vegetative pole of the cell the massing is by no means as pronounced as in *Lymnaea* (Plate I*C*; Fig. 2*A*). Instead, one notes the beginning of a migration of osmiophilic material from the vegetative pole toward the animal pole even in the smaller oöcytes. As the oöcyte enlarges in its envelope the spread becomes more and more pronounced, while the massing in the vegetal pole is less pronounced. By the time the cell has attained three-fourths of its growth, the distribution of the osmio-

philic material is directly comparable with that of the released or shed oöcyte of *Lymnaea* (Fig. 2*B*).

Following the release of the oöcyte of *Physa* from the gland the polarity as marked by the osmiophilic globules is obscured and there is a uniform distribution of the blackened material throughout the cell (Fig. 2*C*). The germinal vesicle is still intact at this stage, and no signs of the beginning of the maturation process have appeared. In this respect the behavior of the dark globules differs strikingly from that observed in *Lymnaea,* where universal and even distribution is not

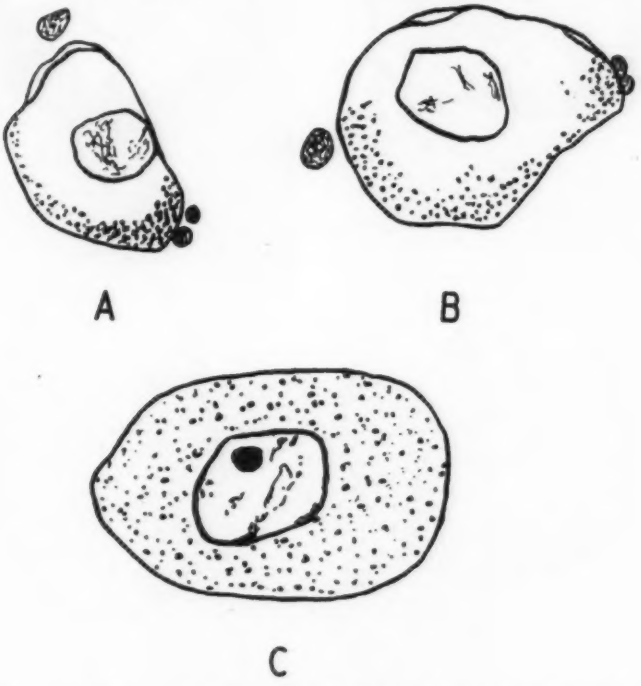

FIGURE 2. Sketches based on camera lucida tracings to show distribution of osmiophilic material during the course of development of the oöcyte of *Physa*. *A*, ovarian oöcyte; *B*, ovarian oöcyte about ⅔ developed. Compare with Figure 1*C*. *C*, oöcyte at the time of release from its envelope. Note the uniform distribution of osmiophilic material, and the fact that the germinal vesicle is still intact.

achieved until after the breakdown of the germinal vesicle. Thus the underlying system in *Lymnaea* appears more "rigid" in its composition, and though the evidence is indirect, one may speculate on the possibility that the ground substance in the oöcyte of *Physa* at the time of its release is in a more fluid physical state than that of *Lymnaea*.

No other obvious differences between species were noted, nor could other indications of the nature of the underlying cytoplasmic patterns be found. In an

effort to throw more light in this direction, living snails containing oöcytes were subjected to centrifugal force so as to displace the visible inclusions of the oöcytes and so further test their organization.

Single snails were placed on non-absorbent cotton in individual centrifuge tubes; the tubes were filled with water; and the snails centrifuged at approximately 1400 × G for thirty minutes. Care was always taken to select snails which were laying prolifically at the time of centrifugation. Of the thirty-six individuals of each species which were centrifuged, twelve were isolated in individual small-sized bowls and retained for subsequent study. The twenty-four remaining specimens were divided into six lots of four each, one of which was sacrificed immediately after centrifugation, and the remainder at intervals of ½ hour, 1 hour, 6 hours, 12 hours, and 24 hours respectively, after centrifugation. Upon excision, the gonads were fixed immediately in Champy's fluid. They were subsequently embedded in paraffin, sectioned, and stained after the Champy-Kull method. The oöcytes in these preparations were then compared with each other and with those of the other lots.

Lymnaea

In the sections of gonads fixed immediately after centrifugation, the contents of the oöcytes are stratified into three zones; one of which is centrifugal and red (yolky), one of which is almost equatorial, relatively clear and faintly pink or

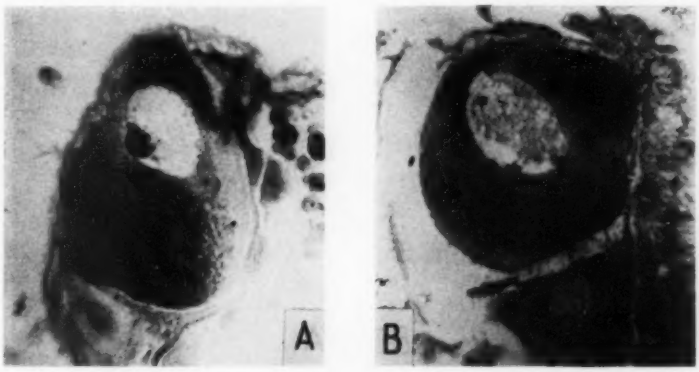

Plate II. Photomicrographs of oöcytes fixed immediately after centrifugation. *A, Lymnaea; B, Physa* (× approximately 450)

colorless (cytoplasm, or hyaloplasm), and one of which is centripetal and black (oil, blackened by osmic acid, and undoubtedly the great mass of the "osmiophilic material" referred to in the previous section). These three zones correspond, no doubt, to those described by Conklin (1910) in eggs of *Lymnaea* and *Physa* which were centrifuged after fertilization and deposition.

The nucleus in all cases lies in the clear or unstained zone, and, when compared with the controls, shows little indication of any displacement. The nucleoli in a great number of cells have been displaced centrifugally in the nucleus, though

this is not always the case. Not all of the yolky material within the oöcyte is massed at the centrifugal pole; some is usually found "trapped" on the centripetal side of the nucleus where it projects into the dark osmiophilic cap (Plate II*A*; Fig. 3*A*).

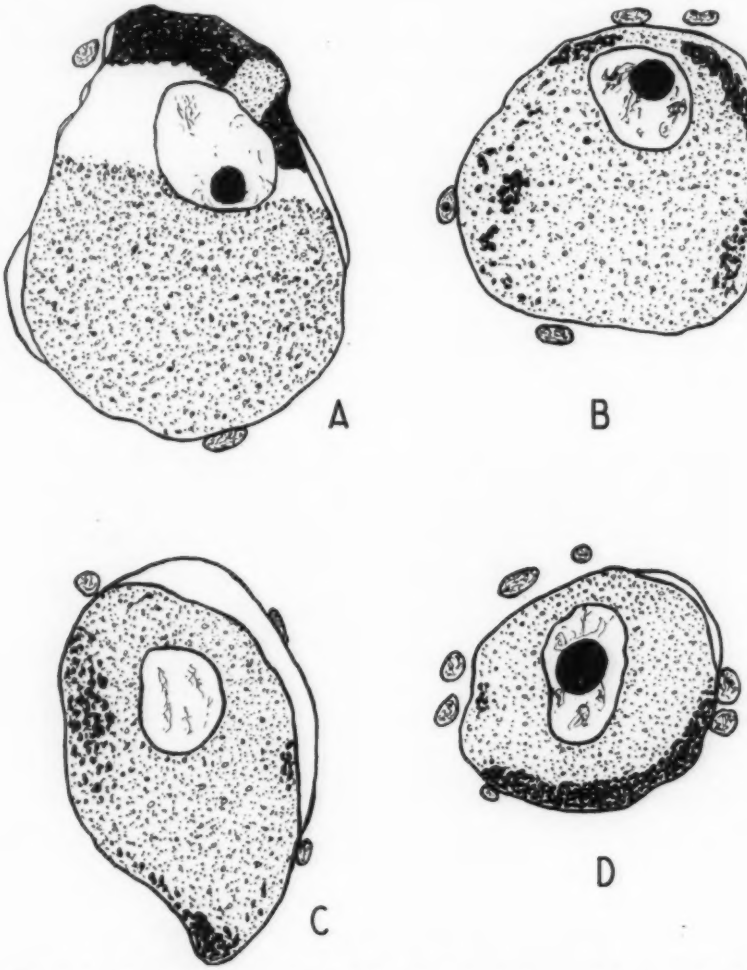

FIGURE 3. Sketches based on camera lucida tracings of oöcytes of *Lymnaea* fixed at different intervals following centrifugation. *A*, immediately after centrifugation, showing distinct layering into a black centripetal zone, clear central zone, and granular yolky zone. Granular area above germinal vesicle represents yolk "trapped" at centripetal end of cell. *B*, oöcyte fixed one-half hour following centrifugation; *C*, cell fixed one hour after centrifugation; *D*, oöcyte from preparation fixed six hours after centrifugation. Compare with Figure 1*A* and *B*.

Since the ovotestis is composed of convoluted tubules or acini, and the oöcytes are located in the walls of these acini, the orientation of the oöcytes with regard to the direction of centrifugal force varies. Stratification bears no relation to previous distribution of materials or visibly expressed polarity. This is the natural expectation based on the results of Conklin, previously mentioned.

The oöcytes in gonads fixed one-half hour after centrifugation already show a breakdown in the stratification. The "clear" hyaloplasmic area is invaded by the yolk, leaving only two of the former zones visible. The osmiophilic centripetal masses are migrating in small loose clumps from the area of former concentration, that material now being restricted to the inner cortical and subcortical regions of the cell. In the outer cortical portion between the remainder of the centripetally massed dark material and the egg surface a thin, nearly clear zone is now visible which extends over the oöcyte from the centripetal pole to a point somewhat below the middle. This zone is found one-half hour after centrifuging but is not apparent in preparations fixed immediately after centrifuging, when stratification is complete (Fig. 3*B*).

One hour after centrifugation the outer cortical clear zone has disappeared and the concentration of the osmiophilic substance has become diffused. Small clumps of the droplets are making their way back to their original position near the vegetative pole. The return path is not directly through cytoplasm and yolky materials but for the most part lies in the peripheral layers of the oöcyte. This phenomenon recalls the work of Lillie (1909) in which a denser "spongy" cytoplasmic core, evidently a part of the ground substance, was demonstrated in the unfertilized eggs of *Chaetopterus*. This center dense area is probably also in part responsible for the "trapping" of some yolky granules at the centripetal end of the oöcyte as described above.

At this stage, the yolky granules are uniformly distributed throughout the egg. The position of the nucleus has not changed, though those nucleoli which were displaced have left the centrifugal pole of the nucleus and have regained their more central location (Fig. 3*C*).

By the end of the sixth hour following centrifugation the oöcyte has, in most respects, regained its typical appearance. One or two isolated clumps of osmiophilic material may still be seen in the course of migration to the vegetative pole, but there are no other indications that the components of the cell have been displaced (Fig. 3*D*). By the twelfth hour all of the oöcytes have reassumed the appearance presented prior to stratification, so that it is impossible to distinguish the centrifuged ovotestis from that of the controls. The same is true for the gonads fixed after twenty-four hours.

Physa

The ovarian oöcytes of *Physa* fixed immediately after centrifugation show four zones. As in *Lymnaea*, there is a concentration of darkened osmiophilic substance at the centripetal pole, a clear band containing the nucleus, and a heavy red, yolky area occupying the greater part of the centrifugal hemisphere of the cell. In addition there may be found a fourth zone which is located at the extreme centrifugal pole. This appears as a small unstained area. In all probability it represents yolky or pigment material which does not react with the Champy-Kull stain,

and which does not separate out under the same centrifugal force in *Lymnaea*. Its position at the centrifugal pole would indicate that it is composed of the densest inclusions of the oöcyte (Plate II*B*; Fig. 4*A*). In general there is less interference with the migration of materials by the germinal vesicle in *Physa* than in *Lymnaea*, and when "trapping" of materials does occur, the obstructed mass is always smaller than that found in *Lymnaea*.

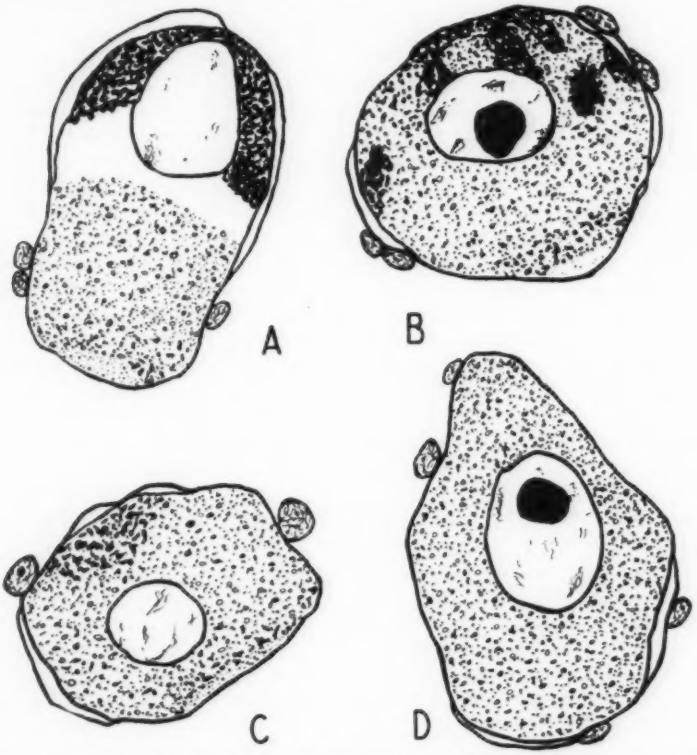

FIGURE 4. Sketches based on camera lucida tracings of oöcytes of *Physa* fixed at different intervals following centrifugation. *A*, immediately following centrifugation; the centripetal pole (black) oriented toward the head of the page, and the small centrifugal band (probably pigment) oriented toward the foot of the page. *B*, one-half hour following centrifugation; *C*, one hour following centrifugation; *D*, six hours following centrifugation. Compare with Figure 2*A* and *B*.

One-half hour after centrifugation, the materials in the oöcyte of *Physa* are fairly well redistributed. The broad, clear, hyaloplasmic band has completely disappeared; the osmiophilic concentration is rapidly breaking up into fragments which are migrating relatively freely through the cell. There is no evidence of the restricted subcortical migration noted in *Lymnaea*. The clear zone at the centrifugal pole has maintained itself, though it is, on the average, smaller (Fig. 4*B*).

One hour after centrifugation the dissociation of the clumped masses of blackened material is nearly complete. A small area is still to be found near what was the centripetal pole, but no other concentrations of osmiophilic material are visible. There is no return of the darkened substance to its previous location near the vegetative pole as in *Lymnaea* but instead these granules or droplets show a uniform distribution throughout the cytoplasm. The clear unstained mass at the centrifugal pole of the oöcyte has become definitely smaller and less distinct (Fig. 4*C*).

After six hours the oöcytes of *Physa* show no evidence of the previous stratification. The materials have been uniformly redistributed and the visible animal-vegetal differentiation which characterized the oöcytes prior to centrifugation has been eradicated. It is not regained in the oöcytes of the gonads preserved twelve and twenty-four hours after centrifugation (Fig. 4*D*).

The eggs of both species laid by the snails isolated after centrifuging were all collected, and their subsequent development was observed over a period of four weeks. In no case was any atypical cleavage or development found; nor did young snails on hatching show any differences in body symmetry from that observed in the normal animal.

On the basis of the above observations, it is possible to draw certain conclusions concerning the oöcytes of *Physa* and *Lymnaea*. We have seen first that the visible materials may be displaced without altering the developmental plan of the eggs. Second, the materials after being stratified, are redistributed rapidly and in the same sequence for each species. Hence we may postulate that some more basic organization or ground substance is present in the oöcyte, as in the deposited egg.

The variance in the manner of redistribution of materials in the two species strengthens the previous suggestion made on the basis of cytological observations of the normal oöcyte, that the physical state of this ground substance differs in the two species. That in *Lymnaea* definitely seems more fixed and not as fluid as that in *Physa*. The "trapping" of relatively large masses of yolk behind the nucleus, the clearly defined peripheral path of migration of the osmiophilic material, the exact reconstruction of the picture found prior to centrifugation, all point to this fact. *Physa* not only shows none of these phenomena characteristic of *Lymnaea* but under equal centrifugal force the materials of the oöcyte stratify into four zones instead of three, the densest inclusions separating out below the yolk in the centrifugal pole. Thus evidently, migration and separation is easier through the ground substance of *Physa* than of *Lymnaea*.

Studies With X-rays

The constancy of the developmental pattern resident in the the ground substance of the oöcyte was next tested by exposure of adult snails, and hence their oöcytes to X-rays.

The snails were placed in small, flat, glass containers and submitted to X-radiation at dosages of 200 r., 500 r., and 1000 r.[2] Fifteen individuals of each species

[2] In all runs reported, the X-rays were produced by a Coolidge type tube, having a water-cooled tungsten target. Dosage was continuous, and was administered at an approximately constant rate of 200 r. per minute.

were exposed at each value and were then isolated and observed at intervals. All egg capsules deposited after exposure to radiation were collected, placed in individual containers of pond water, and observed regularly throughout their subsequent development.

At 200 r. no effect of radiation was evident, either in the behavior of the adult snails or in the development of the eggs recovered. This was the case in both *Physa* and *Lymnaea*.

At 500 r. all of the individuals of *Lymnaea* exhibited a noticeable delay before any capsules of eggs were deposited. No eggs were recovered in any case prior to the fourth day after exposure. This was not true for *Physa* where capsules were recovered as early as twenty-four hours after the radiation of the adult snails.

In both *Physa* and *Lymnaea* the usual number of eggs in each capsule initiated development, though there was some increase in the number of eggs which did not complete their full development. The defects in development appeared usually after gastrulation, about the time of trochophore formation or immediately after. As in the 200 r. group, all snails which hatched were phenotypically similar to the parents.

At values of 1000 r. no egg capsules were recovered from either species.

The above results parallel, to a large extent, observations of Dr. E. V. Enzmann (unpublished). In discussions of his X-ray studies on *Planorbis trivolis*, he reported no success in efforts to cause monsters or reversals in this species. He was unable to find any aberration in early cleavage or in the final body plan of the adult snail, even though he was able to recover eggs from snails subjected to higher dosages than 1000 r.

It would therefore appear that the ground substance of the oöcyte, unless the cell is actually destroyed by X-radiation, is not affected adversely by it, at least in terms of the fundamental factors necessary to institute cleavage and development through gastrulation.

Discussion

A consideration of the results presented here in the light of reports by previous workers will readily bring out certain points worthy of special mention.

In the first place, the contention of Raven and Bretschneider (1942) that the visible elements of the egg are of far greater importance to ontogeny than had been previously thought, would seem to be strengthened. The shifting of the osmiophilic materials in the oöcytes of *Physa* and *Lymnaea* not only after centrifugation but also in the normal development fits in with Raven's view that inclusions shift considerably during development, and that their presence or absence in given regions of the egg or developing embryo is of primary importance. It may be pointed out again, that the shift of the osmiophilic inclusions is always the same in the oöcytes of the same species, while differing between species. This fact may well be tied in with the apparent differences in viscosity of the ground substance of the two species and is even more interesting when one considers that the cleavage pattern also differs, that of *Lymnaea* being dexitropic, while that of *Physa* is leiotropic.

In continuing a comparison of results with those of previous workers, we find some disagreement in the section dealing with centrifugation. Conklin (1910)

and his contemporaries report that following centrifugation the eggs of fresh water snails and other invertebrates stratify into three zones; and oily centripetal, a clear hyaloplasmic, and a centrifugal yolky zone. Raven and Bretschneider, in contrast, report in *Lymnaea* a fourth zone (in their work the third zone) composed of mitochondria, or "α granules," glycogen, and other inclusions, interposed between the clear zone and the centrifugal yolky zone.

The present report is in general agreement with Conklin and disagreement with Raven and Bretschneider. True, in *Physa* a fourth zone, not reported by Conklin, was found at the extreme centrifugal pole, but this is not of especial note since in these studies the oöcytes were subjected to far greater centrifugal force than was used by Conklin. Hence some additional separation of pigment or other denser inclusions from the yolk might normally be expected. However in *Lymnaea* no trace of Raven and Bretschneider's "fourth zone" between the clear and yolky bands could be found. This may be due in part to the fact that these workers in turn used somewhat higher values of centrifugal force than did the present writer. On the other hand, Raven himself reports that in certain of his preparations fixed in Champy fluid the "α granules" cannot be made out. This may rationalize the difference, since all of the preparations used in the present study were Champy fixed. Thus it is possible that the "fourth zone" was present but did not show up.

Why Champy fixation does not demonstrate this zone is an interesting cytological question. The works of Harvey (1936, 1938) using the eggs of echinoderms and of Clement (1938) using eggs of *Physa* certainly contribute good evidence in behalf of Raven's suggestion that such a zone should be largely made up of mitochondria. But a Champy-Kull stain following Champy fixation should demonstrate mitochondria. Raven and Bretschneider can offer no explanation for the discrepancy, nor is it possible for the present writer to do so on the basis of this study.

Beyond this difference the results of the sections on centrifugation and X-radiation follow closely the findings of former workers, which demonstrated that the underlying pattern of the deposited egg cannot be altered by centrifugation or similar forces. In this respect the ovarian oöcyte is evidently no different.

Summary and Conclusions

The developing oöcytes of two species of fresh water snails, *Lymnaea stagnalis* and *Physa gracilis*, are studied cytologically and experimentally through the use of X-radiation and centrifugal force. As a result of the cytological studies, certain differences in the pattern of distribution of visible inclusions are pointed out. These are specific and constant within each species, but differ between species.

The constancy of pattern within the ovarian oöcytes was tested in both species by centrifuging entire snails. Displacement and stratification of the visible cellular inclusions in the oöcytes was produced, but no aberrations in subsequent development resulted. It is concluded that a fundamental ground substance is present in the oöcyte which is not affected by the stratification of visible inclusions. The manner in which the inclusions migrated from their artificially induced stratified locations was observed, and it is postulated that the ground substance of the oöcyte of *Physa* is in a more fluid physical state than that of *Lymnaea*.

Further tests on the constancy of pattern were made by exposure of snails of each species to varying dosages of X-radiation and study of the development of

eggs laid by them subsequently. It was found that as dosage increased there was a decrease in the number of eggs which were able to complete their embryonic development. No decrease was noticed, however, in the number of eggs which were able to initiate their development. It is concluded that the cytoplasmic pattern or ground substance inherent in the oöcyte is unaffected by X-radiation of dosage values below killing intensity.

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THE RELATION OF BLOOD OXYGEN TRANSPORT TO RESISTANCE TO ANOXIA IN CHICKS AND DUCKLINGS [1]

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The relatively greater resistance of the new-born and young animals to complete anoxia in comparison with that of the adult animal has been studied by Himwich et al. (1941) who have advanced the hypothesis that differences in the aerobic and anaerobic energy release in the young and adult brains are the probable cause of the differences in resistance to anoxia. The newborn and young of many species of animals have been exposed to 100 per cent N_2, He, and CO_2 and have proved to be remarkably resistant to complete anoxia in comparison to older individuals of the same species (Fazekas et al., 1941). Since there is a total lack of oxygen in the blood stream under conditions of total anoxia and since the brain is the organ most susceptible to anoxia the oxygen requirement of the brain of newborn and young animals has been studied in comparison to the energy requirement of the brain of older animals of the same species in order to find some possible basis for the greater resistance of the young and newborn to anoxia. Himwich et al. (1941) and Chesler and Himwich (1944) have demonstrated that the anaerobic energy release in the brain of the newborn is relatively greater, in proportion to the total energy requirement of the brain, than in older individuals of the same species. After birth there is a rapid increase in energy requirement as measured by the amount of respiration carried on in the brain (Tyler and von Harreveld 1942). This increase is met, chiefly, by increase in the aerobic energy release. In the newborn the total energy requirement is low and a relatively larger proportion of the requirement is met by anaerobic energy release. The survival of the newborn and young under conditions of total anoxia is adequately explained on the basis of the relatively great anaerobic energy release in the brain of the young and newborn compared to the total energy required by the brain at this stage of development to maintain the functional integrity of the organism.

Although Quastel and Wheatley (1932) demonstrated differences in the energy requirement of surviving brain slices of cat and rat brains—the rat brain had a higher energy requirement as evidenced by greater oxygen consumption of surviving brain slices—the possibility remains that in the adult animal species differences in survival at high altitude under conditions of incomplete anoxia may be explained, in part, on several bases beside differences in brain metabolism. One of these factors is differences in oxygen transport by the blood.

[1] This work was supported by a grant from the John and Mary R. Markle Foundation to the Pathology Department. The anti-coagulant used in these experiments was "Lequaemin" supplied by Roche-Organon Inc., Nutley, New Jersey. Some of the chicks used in these experiments were given to the University of Arkansas by the Arkansas Hatchery, Little Rock, Arkansas. Research paper 595, Journal Series, University of Arkansas.

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Rostorfer and Rigdon (1945) were able to show that malarial infection greatly reduced survival time of ducks and chicks at simulated high altitude. The effect of a severe malaria infection on the survival time was marked by the third day after the birds had been inoculated with the malarial organism. At this time there was only a small percentage of red cells infected, but a shift in acid-base balance and a reduction in the oxygen carrying capacity were developing. In the study of the effect of malaria on survival of birds at high altitude a specific difference in resistance to high altitude anoxia between ducks and chicks was reported. This difference in resistance was still evident on the third day after inoculation with malaria, although there was decreased resistance in each case.

These earlier experiments suggested to the authors that specific blood differences might be related to the specific resistance to anoxia in the case of young ducks and chicks. Accordingly, a study was made of the oxygen capacities, the oxygen dissociation curves, the amounts of hemoglobin present in blood of young and adult ducks and chickens in relation to their resistance to anoxic anoxia.

Materials and Methods

White Pekin ducks and chicks of mixed breed, obtained from a commercial hatchery, between 3 and 21 days old were subjected to continuous decompression until convulsions and death occurred in a decompression chamber as previously described by Rostorfer and Rigdon (1945). Five to ten birds of the same age and species were observed during one decompression. In all experiments the rate of decompression was approximately the same with only a slight variation. The rate during the first four minutes of simulated ascent was between 1,700 and 1,800 feet per minute, between 700 and 900 feet per minute during the next 15 minutes, and 400 feet per minute during the next 40 minutes. After reaching a simulated altitude of 38,000 feet the rate of climb was such that during the next 12 minutes the altitude increased to only 40,000 feet. The altitude was recorded every 4 minutes during the decompression. These data were plotted against time and the total area under the resulting curve up to the time of death of the bird was calculated for each bird. The total area under the curve may be taken to represent the resistance of the bird to anoxia. The unit of resistance was expressed as the altitude in feet times the duration in minutes and was expressed as foot-altitude minutes.

Ducklings and chicks in groups of 7 to 12 individuals the same age were placed in the tank and decompressed until they were dead in the manner described. The ages of the various groups were 3, 6, 10, 15, and 20 days old. The average resistance to incomplete anoxia at simulated high altitude was obtained by analysis of data found by decompressing several age-groups of birds of each species. The number of birds in each separate decompression was determined by the size of the individuals.

Blood samples were obtained by heart puncture from 3 to 4 ducks or 6 to 12 chicks of the same age and pooled. Coagulation was prevented by adding 1 ml. of a 1 per cent solution of the sodium salt of heparin to each 9 ml. of blood. All red cell counts and hemoglobin and gas analyses were carried out on the pooled samples.

Oxygen capacities were determined by analysis of blood equilibrated in Barcroft tonometers with air at 38° C. Analyses were made by means of the Van Slyke manometric apparatus.

Oxygen dissociation curves were calculated for the pooled samples by equilibrating blood at a constant CO_2 tension of 31 mm. Hg and at varying oxygen tensions. Three gas mixtures were used containing 3.4, 7.44, and 11.86 per cent oxygen which corresponded to oxygen tensions of 24 ± 0.5, 53 ± 0.5, and 83 ± 0.5 mm. Hg, respectively. Analyses carried out on blood equilibrated at these three oxygen tensions gave data from which the oxygen dissociation curves were calculated by the use of the formula $y = \frac{100\ Kx^n}{1 + Kx^n}$, when the oxygen capacity of the blood was known (Hill, 1910).

Hemoglobin determinations were made on each pooled sample by the use of the Schulte and Elvehjem (1934) colorimetric method. Hemoglobin was determined, also, by calculation from the oxygen capacity. These data indicate the amount of functional hemoglobin.

Cell counts were made in the usual manner on the pooled blood and the color indices were calculated by dividing the grams of hemoglobin calculated from the oxygen capacity by the total red cell counts.

Results

In order to determine the specific resistance of young and adult ducks and chickens to the incomplete anoxia produced at simulated high altitude a series of decompression experiments were carried out in the manner described. The data represented by Figure 1 show the decreasing resistance to anoxia with increasing age. The three-day old birds of both species had the greatest resistance. There appeared to be no change in resistance between the 15- and 20-day old birds in either species. There was, however, definitely greater resistance in the duck than in the chick at all ages compared. Adult birds were not studied but there are reasons to assume that the adult duck would be more resistant to anoxia than the adult chicken.

These data indicate that the newly hatched and young birds of the same species are more resistant to incomplete anoxia produced at simulated altitude. This corresponds to the resistance of young and newborn mammals studied by Himwich and others. The data indicate, also, a specific difference in resistance to incomplete anoxia between ducks and chicks. Ducks 20 days old decompressed in the usual manner succumbed at altitudes between 34,000 and 36,000 feet, while chicks 20 days old were all dead at 27,000 feet when the decompression was at the same rate as that for the ducks.

There is little doubt that specific differences in oxygen capacity and color index exist among animals. This difference might possibly bear some relation to resistance to anoxia. The oxygen capacities and color indices were determined for all blood samples. Determination of oxygen capacities of duck and chick blood revealed that the duck blood had considerably greater oxygen capacity. Duck blood from 20-day old birds had an average oxygen capacity of 12.27 volumes per cent. Chick blood from birds the same age averaged only 10.47 volumes per cent.

The color indices of the 20-day old duck varied around 3.6×10^{-11}. Rostorfer and Rigdon (1946) found that the color indices gradually increased with age of the young ducks. The color index of chicken blood is approximately 3.1×10^{-11} and there seemed to be no increase in the color index with age as in the duck. The

color index of blood taken from the adult chicken was 3.3×10^{-11}. There was an appreciable difference in total red cell counts between the duck blood and the chick blood. The average count for the pooled blood of the chick was 2.2 million and the average count of the pooled blood of the duck was 2.6 million per cubic millimeter.

The chick blood in comparison to the duck blood had fewer red cells, decreased color index, and lower oxygen capacity.

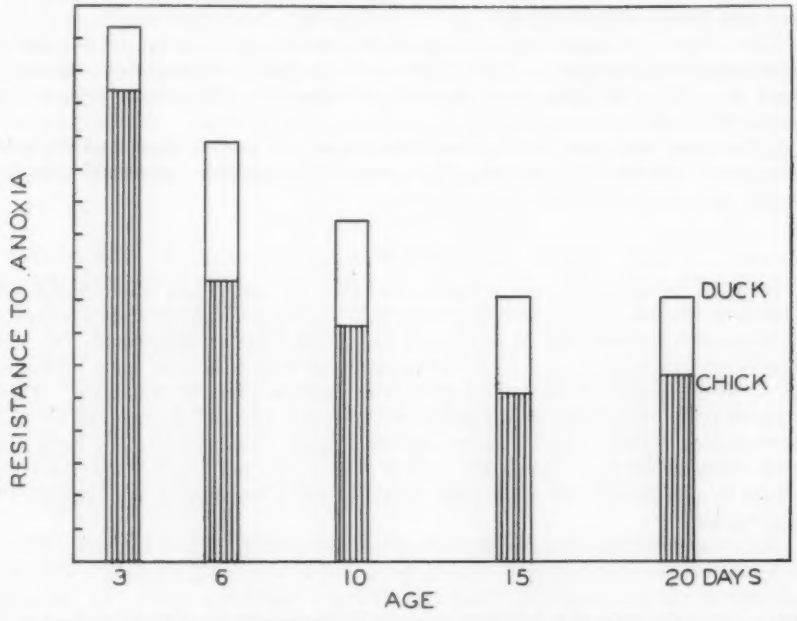

FIGURE 1 is a graphic representation of the data on the study of relative resistance of chicks and ducks to simulated altitude. Resistance is expressed in feet of altitude times the duration at altitude in minutes. Each age group represents the average data of 10 to 20 ducks or chicks.

A considerable difference between the amount of hemoglobin determined colorimetrically and that calculated from the oxygen capacity was found for young and adult ducks by Rostorfer and Rigdon (1946). A similar study was carried out on young and adult chicks. The comparison is made in Figure 2 which represents the relationship between the amount of hemoglobin determined colorimetrically and the amount of hemoglobin calculated from the oxygen capacity. The linear expression was obtained by determining the amount of hemoglobin colorimetrically and by calculation from the oxygen capacity of a diluted, a normal, and a concentrated sample of the same pooled blood. Cell counts of the three samples, dilute, normal, and concentrated, were made and the color index of each determined. There was usually close agreement between the color indices of the three samples. The young chicks and ducks carry less oxygen per gram of hemoglobin than the

adults based on the colorimetric hemoglobin. There was a slower shift toward the adult relationship with increasing age in the duck than in the chick. Thirty-day old chicks were found to have hemoglobin which corresponded to that found in the hen. The linear relation between oxygen capacity and the acid hematin of the adult chicken blood was the same as that of the young duck.

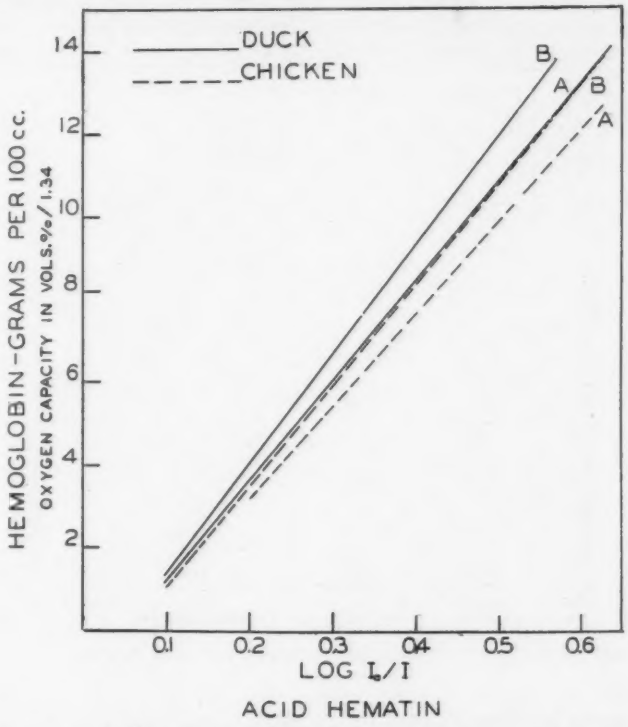

FIGURE 2 is a graphic representation of the relationship of the amounts of hemoglobin calculated from the oxygen capacity in comparison to the amount of hemoglobin determined by photo-electric colorimetry in the blood of young (*A*) and adult (*B*) domestic ducks and chickens.

The equilibrium between hemoglobin of bird blood and oxygen at various oxygen tensions has been investigated by Wastl and Leiner (1931) whose work indicated that blood of the duck and of the pigeon does not have typical oxygen dissociation curves. Redfield (1933) points out that such curves for blood containing hemoglobin had not been described previously but were characteristic of the blood of invertebrates. Christensen and Dill (1935) were not able to confirm the work of Wastl and Leiner, but found that the blood of ducks and other birds developed equilibria between hemoglobin and oxygen in the classical manner except at very low oxygen tensions. This discrepancy they attributed to a systematic error of equilibration. Rostorfer and Rigdon (1946) found considerable difference be-

tween the dissociation curves for young and adult ducks. The blood of the adult birds gave a typical sigmoid curve while that of the young ducklings had a greater oxygen uptake at lower oxygen tensions than would be indicated by calculation from points of equilibrium at higher oxygen tensions. However, the deviation was slight.

Bloods of young and adult chickens were investigated in similar manner. Figure 3 compares the oxygen dissociation curves of adult ducks and chickens. There was a considerable difference between the oxygen uptake of the blood of the

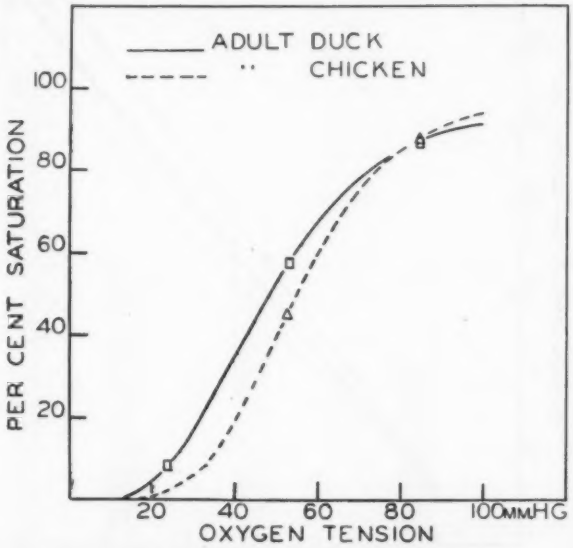

FIGURE 3 compares the oxygen dissociation curves for the adult duck and chicken at a CO_2 tension of 31 mm. Hg.

adult duck and the adult chicken at various oxygen tensions. The adult duck blood had about the same percentage of saturation at ordinary oxygen tensions but was capable of carrying a larger amount of oxygen than chick blood at low oxygen tensions.

Figure 4 compares the dissociation curves of the blood of the adult and of the young chicken. Although the curve for the blood of the young chick represents the data obtained from one sample of pooled blood only, it is reasonable to assume that it is representative. In general, the blood of the young chick appeared to have a greater percentage of saturation at all levels of oxygen tension than the blood of the adult chicken. In contrast to the blood of the adult duck, the blood of the young chick had a higher percentage of oxygen saturation at higher oxygen tensions but a slightly lower per cent of saturation at low oxygen tensions.

In general, the blood of the duck in comparison to chicken blood had greater oxygen capacity, contained more functional hemoglobin, and showed less dis-

crepancy between the colorimetric hemoglobin and the hemoglobin calculated to be present from the oxygen capacity. Duck blood had a larger number of cells and a higher color index than chicken blood. The chemical behavior of duck blood in comparison to chicken blood, as regards the oxygen dissociation curves, indicated that the duck blood would be more efficient in the transport of oxygen under conditions of incomplete anoxia. The blood of the duck in comparison to that of the chick would appear to be more capable of maintaining the brain and other vital organs in a viable condition for a longer time under conditions of simulated high altitude and resulting anoxia.

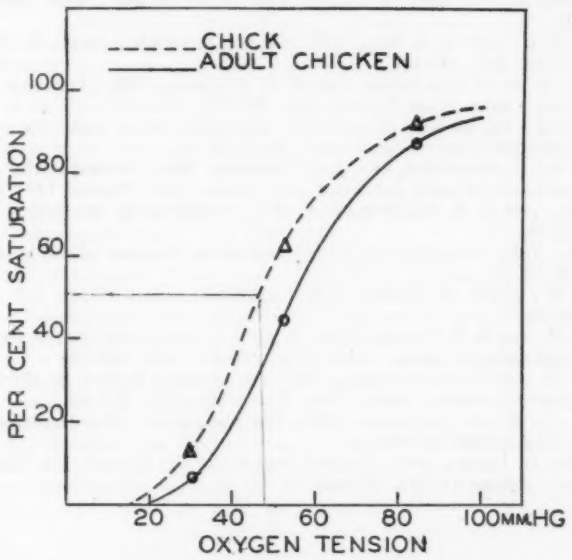

FIGURE 4 compares the oxygen dissociation curves of young and adult chicken blood at a CO_2 tension of 31 mm. Hg.

It is not unreasonable to assume that some of the difference in specific resistance to anoxia of the duck and the chick is caused by the greater efficiency of the blood of the duck at low oxygen tensions. However, a study of the anaerobic and aerobic energy release compared to the total energy requirement of the brain of the two species in question has not been made as yet. One might well assume that there are specific differences in brain metabolism.

SUMMARY

A study of the resistance of ducks and chicks to incomplete anoxia produced at simulated altitude is reported. The newly-hatched and young birds had greater resistance than older birds. The duck was found to be more resistant than the chick to incomplete anoxia at all ages studied.

A study of the oxygen carrying capacities, total red cell counts, color indices, the amount of hemoglobin, and the oxygen dissociation curves of the blood of the duck and the chick is reported.

The blood of the duck has greater oxygen capacity, higher total red cell count, higher color index, more hemoglobin and a higher percentage of saturation of the blood with oxygen at low oxygen tension when compared with blood of the chicken.

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THE FOOD-VACUOLE IN *PARAMECIUM*[1]

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INTRODUCTION

The food-vacuole has been more extensively studied in *Paramecium* than in any other organism. There is, however, still much diversity of opinion concerning the processes involved in its formation and movement and in the changes that occur in it. These phenomena will be considered in the following pages.

MATERIAL AND METHODS

Four species of *Paramecium*[2] were used in the following observations, namely, *caudatum, multimicronucleatum,*[3] *aurelia,* and *trichium.* All the specimens used were obtained from pure cultures maintained in the laboratory.

[1] I am indebted to Dr. W. J. Bowen for very efficient assistance in some of this work, and to the artist John S. Spurbeck, for expert service concerning the figures.

[2] Dr. D. H. Wenrich very generously identified the species used.

[3] The name *"multimicronucleatum"* is so long and unwieldy that the abbreviation, *nucleatum,* will hereafter be used in place of it.

Paramecia are usually so active that it is very difficult to study them under high magnification. Various methods have been used to quiet them, e.g., addition of narcotics (Bills, 1922), addition of yeast-cells stained with congo red (Buck, 1943), increase in viscosity (Marsland, 1943; Moment, 1944; and others). All these methods and also reduction in temperature were tried. The best results were obtained with the second method and some modifications of it.

It was found that locomotion can readily be stopped without apparent injury by means of narcotics or by increasing the viscosity of the culture fluid, but that when locomotion ceases under these conditions, feeding does also. It was also found that while movement decreases greatly with decrease in temperature, it does not decrease sufficiently to make observation under high magnification practicable until after the paramecia have been actually frozen and killed.

Nearly all the observations were made on preparations made as follows: A drop of solution containing numerous paramecia from a young, vigorous culture was mounted between two small ridges of vaseline on each of several slides. Yeast-cells stained with congo red (Buck, 1943) were added to some, chinese ink or carmine to others, and an abundance of zooglea and a little neutral red or nile blue to still others. Then all were covered with cover-glasses and sufficient fluid removed from some to flatten the paramecia considerably. All the preparations were kept in a damp chamber when not under observation.

The paramecia in these preparations usually lived for several days. They were very active at first but they soon became quiet, although it sometimes required 24 hours or more, and in some of the preparations they became so quiet that given individuals could be studied continuously for several minutes, even under an oil-immersion objective. Nearly all the quiet paramecia, even those which had been flattened, fed vigorously. In some of these, especially those which were flattened, the structure of the feeding apparatus, the activity of the cilia and the movement of particles in it, and the formation of the food-vacuoles could be clearly seen.

It was found that the paramecia from young, rapidly growing cultures become quiet much more readily than those from old declining cultures. This is doubtless, at least in part, due to difference in the hydrogen-ion concentration of the cultures. At any rate, it was observed that if a trace of alkali is added to fluid from young cultures the activity of the paramecia in it increases greatly and that if a trace of acid is added to fluid from old cultures the activity of the paramecia in it decreases.

The Feeding Apparatus

Numerous observations were made on the feeding apparatus in the four species of *Paramecium* listed above. The following conclusions were reached: The feeding apparatus is essentially the same in structure in the four species studied. It consists of a shallow ciliated groove (the oral groove) which extends from the anterior end to slightly beyond the middle of the body, a ciliated depression (the vestibulum) at the posterior end of the groove, a ciliated tube which extends from an opening in the floor of the depression (the mouth) backward into the body, and some fibers which extend from the distal end of the tube toward the posterior end of the body.

The tube is called pharynx by some, cytopharynx, gullet or esophagus by others, and a portion of it pharynx and the rest esophagus by still others. I shall call it

pharynx. The pharynx can be seen distinctly in living specimens. It extends from the mouth directly toward the center of the body a short distance, then turns backward sharply and proceeds parallel with the surface of the body for some distance, then turns sharply again but nearly at right angles to the first turn and ends almost immediately in an elliptical opening which leads into the forming food-vacuole which I shall designate "esophageal sac" (Fig. 1).

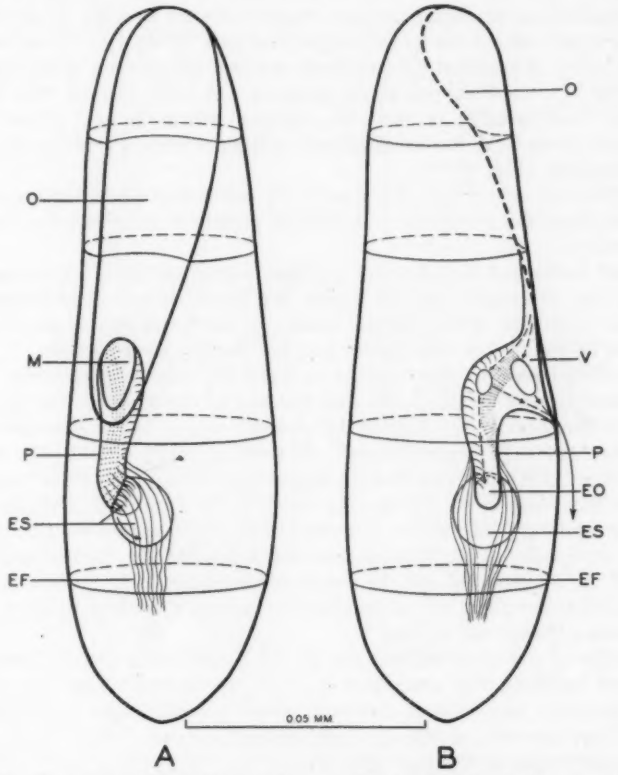

FIGURE 1. Camera outline of *Paramecium aurelia* showing the feeding apparatus and its relation to the rest of the body. *A*, oral groove and mouth facing upward; *B*, oral groove and mouth facing to the right; *O*, oral groove; *M*, mouth; *V*, vestibulum; *EO*, esophageal opening; *P*, pharynx; *ES*, esophageal sac; *EF*, esophageal fibers.

Nirenstein (1905) and others maintain that the mouth is a fixed oval opening. But Frisch (1937) says it "is a narrow slit bounded by a raised, thickened border in the form of an elongated oval, somewhat like lips" and that it is sometimes closed. His observations were made on an exconjugant individual, beginning immediately after it had separated from its mate and continuing for one and one-half hours. I have seen the mouth under the oil-immersion objective in many individuals under

various conditions but not in any immediately after conjugation. In all these, the mouth was continuously distinctly oval in outline, and I did not see any indication of change in form or size. It may well be that the phenomena observed by Frisch occur only for a few hours after conjugation.

If a paramecium is so oriented under the cover-glass that the anterior end is directed from the observer and the oral groove is to the left and faces upward, one can see down through the mouth into the proximal end of the pharynx and one can see the esophageal sac extending at right angles from the opening at the distal end. If the paramecium now rotates on its longitudinal axis 90° to the left, one can see the sharp curve in the pharynx at the proximal end and one can see down through the esophageal sac into the distal end of the pharynx. A more distinct view of this sac (the forming food-vacuole) is, however, obtained when the oral groove is to the right and faces downward, for the distal end of the pharynx is now nearer the upper surface of the body (Fig. 1).

The esophageal sac consists of a thin elastic membrane which separates the content of the sac from the cytoplasm. It changes greatly in form and size as the food-vacuole develops.

The inner surface of the pharynx contains numerous cilia. This can be seen clearly in living specimens, but no details can be made out concerning their arrangement or their size or their action, except at the distal end where a number of long cilia can be seen to beat vigorously into the forming food-vacuole.

Gelei (1934) found, in observations on fixed and stained paramecia, that there are in the pharynx two bands of cilia and that one of these bands, the "penniculus", extends from the anterior left edge of the pharynx nearly to the posterior oral edge and the other (called "Vierermembran") from the anterior aboral edge to the posterior oral edge. He maintains that the former usually contains eight rows of short cilia and the latter four rows of long cilia which at the distal end of the pharynx extend into the forming food-vacuole. Lund (1933, 1941) agrees with Gelei in reference to the composition, the location and the extent of the former and the composition and the location but not the extent of the latter. He holds that the latter does not extend to the distal end of the pharynx but that there is at this end a "pouch" which contains a "heavy tuft of cilia."

The results of my observations are in full accord with Gelei's contention. I could not see anything that resembled a pouch at the end of the pharynx, but I could see distinctly long cilia at this end, which extended into the forming food-vacuole. These however, clearly appeared to be at the end of the "Vierermembran" not in a partially separated pouch (Fig. 2).

Bozler (1924) maintains that there are attached to the right wall of the pharynx about half-way up, some ten long fibers which extend nearly to the posterior end of the body, and that these fibers are fixed in position, fairly rigid and in the same plane. He calls them "Schlundfaden" and says similar fibers occur in other protozoa and that Schuberg (1890) first discovered them in observations on *Stentor*.

Gelei (1934) and Lund (1933, 1941) confirm Bozler in reference to the presence of long fibers attached to the pharynx in *Paramecium* but they do not agree with him in the contention that the fibers are fixed and in one plane. Gelei holds that they are attached to the "right wall" of the pharynx but near the proximal end and that they are not all in one plane. Lund maintains that they are attached near the distal end of the pharynx on all sides and are not fixed. He says (1933, p. 55):

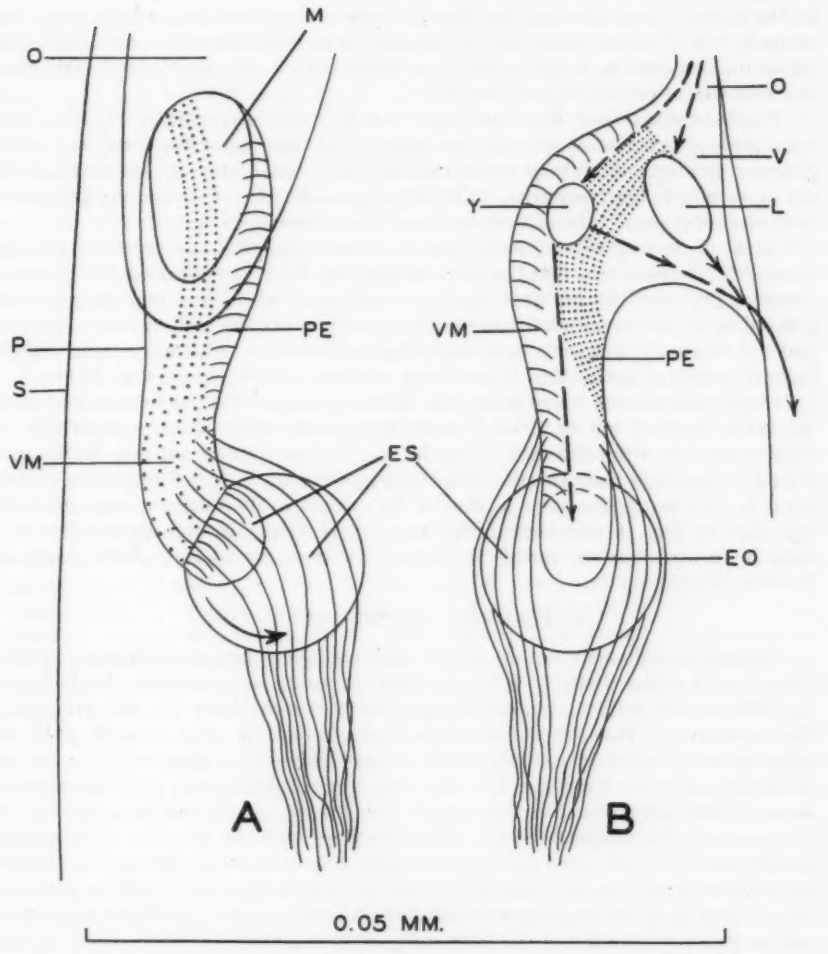

FIGURE 2. Camera outline showing the structure of the feeding apparatus in *Paramecium aurelia*. *A* and *B*, two views, one at right angles to the other. *O*, oral groove; *V*, vestibulum; *M*, mouth; *P*, pharynx; *EO*, esophageal opening; *ES*, esophageal sac; *EF*, esophageal fibers; *PE*, penniculus; *VM*, "Vierermembran"; *Y*, yeast-cell; *L*, a large particle; *S*, surface of the body; broken lines, paths taken by particles during the process of feeding; arrows, direction of movement.

The cilia which project into the esophageal sac from the end of the pharynx are doubtless part of the "Vierermembran." There are, of course, many more than are represented.

"Frequently in stained animals they are wrapped almost around the food vacuoles. In the living animal they can be observed only a short way beyond the gullet, but certainly not all in one plane and are capable of some movement. All morphological evidence points to a lax condition of these fibrils, with their distal ends free." He calls them "postesophageal fibrils".

Lund maintains that there are also "five or more heavy fibrils" which extend from their attachment at the anterior edge of the opening at the distal end of the pharynx, to the posterior edge where each ends in a "large granule" but that they can not be seen in living specimens. The larger granules, Lund thinks, are part of the neuromotor system. He calls these fibers "paraesophageal fibrils".

I have made extensive observations on numerous living specimens of the four species of *Paramecium* listed above, concerning fibers attached to the pharynx. Some of the observations were made on specimens which had been greatly compressed under the cover-glass, others on specimens stained with various vital dyes and still others on specimens in various stages of inanition. All were made with the highest grades of optical and illuminating systems (Mast and Bowen, 1944).

I could not see any fibers across the distal opening of the pharynx in any of the specimens studied, but in a few I could see several fibers which appeared to extend posteriorly from this end. I could not, however, make out any details as to their attachment or their length. This indicates that the index of refraction of these fibers is very nearly the same as that of the cytoplasm in which they are imbedded and that in this respect they differ from those found in the Peritricha for in these organisms they can readily be seen in the living state (Mast, 1944; Mast and Bowen, 1944).

The Formation of the Food-Vacuole

Paramecia ordinarily do not ingest anything when they are swimming actively (Mast and Lashley, 1916). When they are at rest or are swimming slowly in contact with a solid surface, the oral cilia produce a current down the oral groove into the vestibulum. This current contains in suspension all sorts of small solid particles many of which are carried into the vestibulum, but most of these are immediately carried out again. The rest pass through the mouth into the pharynx; some of these are thrown back out again, the rest and some fluid pass through the pharynx into the esophageal sac. Here the fluid with the particles in suspension usually rotates rapidly, and as more and more substance enters the sac, the particles in it become more concentrated and the sac becomes larger, and finally a portion of it separates from the pharynx as a full-fledged food-vacuole. All these phenomena can be seen readily and there is little disagreement concerning them; there is, however, marked diversity of opinion concerning the processes involved. These will be considered in detail under several headings in the following pages.

Selection of particles.

Metalnikow (1907, 1912) concludes that while paramecia ingest all sorts of small particles they take more of those which are digestible than of those which are not. This conclusion is supported by Bozler (1924), Losina-Losinsky (1931), Bragg (1936a, 1939), and others. It is consequently fairly certain that paramecia can differentiate between various small particles. There is, however, little known concerning the factors involved.

Bozler (1924) maintains that selection is usually made at or near the entrance to the pharynx by the response of individual cilia to contact with particles which have been carried into the vestibulum by the oral current, i.e., that if a particle comes in contact with a cilium, the cilium responds either by throwing the particle into the pharynx or into the outgoing current, depending upon the physical characters of the particle. He says (p. 189): "Man ersieht . . . dass das Hineinbefördern der Nahrungsteilchen nicht durch ein planmässiges Zusammenwirken der Cilien geschieht, sondern dass jede Cilie auf Grund der Reize reagiert, die sie direkt von dem Nahrungskörper erhält." He maintains, however, that particles which have entered the pharynx are sometimes forced back out, even after they have nearly reached the distal end, but that this occurs only if the particles are large enough to fill the lumen of the pharynx. Bozler's contention that selection in *Paramecium* is dependent upon physical properties is supported by Schaeffer (1910) in observations on *Stentor*. Nelson (1933), on the contrary, presents convincing evidence that selection, in at least some ciliates, is dependent upon chemical properties.

Losina-Losinsky (1931) holds that discrimination between particles depends upon the action of cilia controlled by a central coordinating system, rather than upon the direct response of individual cilia to contact with the particles and that the stimulating agent is chemical rather than physical. He holds that acceptance of a particle is essentially the result of a chemopositive response and rejection to that of a chemonegative response.

I made numerous observations on the movements of particles in the feeding apparatus of *Paramecium caudatum, P. nucleatum, and P. aurelia.* Most of these observations were made under the oil-immersion objective with specimens variously oriented (Fig. 1). Some of the specimens were in culture fluid which contained numerous bacteria and various other solid particles and others were in culture fluid to which chinese ink, carmine, wheat starch and yeast-cells stained with congo red had been added respectively. No difference was observed in the three species studied.

When the paramecia were actively feeding, many particles of all sorts found in the surrounding medium were carried into the vestibulum, but only a small proportion of these passed on through the mouth into the pharynx. The rest passed out again—some immediately, some not until after they had circulated around in the vestibulum for a time. Nearly all the particles which entered the pharynx were small. A few of those and all the larger ones which entered, passed rapidly on into the esophageal sac without any indication of retardation on the way. The rest immediately after they had passed through the mouth, plunged, one at a time, into the band of cilia (the "Vierermembran") on the the aboral wall of the pharynx. Here they stopped a moment and then either passed back out through the mouth and the vestibulum or directly on into the esophageal sac (Fig. 2*B*).

It consequently seems to be the cilia in the "Vierermembran" near the proximal end of the pharynx which, after momentary contact, either throw the particles back out of the pharynx or down toward the esophageal sac. I have no evidence which indicates what it is that decides the fate of these particles after they have come in contact with these cilia. I have seen many stained, dead yeast-cells of all shapes and sizes rejected after they had come in contact with them and I have seen many of the same shapes and sizes accepted. There appeared to be neither rhyme nor reason in the process under these conditions.

The results presented indicate, therefore, that selection among the larger particles takes place in the vestibulum and selection among the smaller ones in the proximal region of the pharynx and the vestibulum; but they do not illuminate the problem concerning the nature of the stimulating agent involved in the selection of either.

Some of the particles ingested were surprisingly large. Several specimens of *Chilomonas paramecium*, 18 μ long and 10 μ wide, and starch grains, 16 μ in diameter, were seen to pass through the pharynx into the esophageal sac (Fig. 2). Bozler (1924) also found that paramecia sometimes ingest extraordinarily large particles. He found starch grains 11 μ in diameter in the food-vacuoles. The ingestion of particles as large as these requires great extension of the pharynx, for it usually is, in the smallest region, only five to seven micra in diameter. This shows that the pharynx is far from being as rigid as it appears to be.

Bills (1922) and Bozler (1924) maintain that particles are often rejected after they have passed well on down the pharynx, sometimes nearly, if not quite, to the esophageal sac. They consequently imply that selection of food may take place anywhere in the pharynx. Lund (1933, 1941) seems to hold that the particles which have entered the pharynx collect in front of a sort of fibrous screen at the mouth of the esophageal sac and then gradually slip through "one by one" into it and that selection takes place here, at least in part.

I have seen hundreds of particles of all sorts pass through the pharynx into the esophageal sac under various conditions, but I have never seen one rejected after it had passed beyond the first bend in the pharynx; nor have I ever seen any indication of decrease in the rate of movement between this bend and the sac.

The enlargement of the esophageal sac and the increase in the concentration of particles in it.

When a food-vacuole forms by constriction of the esophageal sac, a portion of the sac remains as a membrane over the distal opening of the pharynx. This membrane bulges slightly out into the cytoplasm and thus forms a shallow new esophageal sac. If there is active ingestion, this shallow sac rapidly becomes deeper and finally nearly spherical in form. It contains relatively much fluid and few particles at first, but as it increases in size the concentration of particles increases (at first slowly then more and more rapidly) until the sac often appears to be almost filled with them. The long cilia which extend from the end of the pharynx into the sac beat vigorously; this causes the particles in the sac to vibrate actively and its entire content to rotate. Rotation usually continues until the sac is closed; but sometimes it ceases long before the sac is closed and then only those particles vibrate which are in close contact with the cilia at the mouth of the sac. This is doubtless due to increase in the viscosity of the fluid in the sac. The quantitative relation between fluid and solid particles in the newly formed food-vacuole varies very greatly. Under some conditions the vacuole appears to be entirely filled with fluid, no particles whatever being visible even under the highest magnification, while under others, as stated above, it appears to be almost entirely filled with particles.

Bütschli (1889) and Horning (1926) contend that the content of the pharynx is in direct contact with the cytoplasm, i.e., that no membrane intervenes at the distal opening and that the droplets (vacuoles) which pass from the pharynx into the

cytoplasm are surrounded merely by a surface film. Bütschli seems to think that this continues, but Horning holds that in the cytoplasm, membranes form at the surfaces of the droplets. Gelei (1934) concludes that the content of the pharynx is separated from the cytoplasm by a definite membrane which persists as the esophageal sac enlarges, and that this membrane is porous; for he observed in stained whole mounts, that the cytoplasm adjoining it was strongly stained, whereas that adjoining the pellicle at the surface of the body and that adjoining the wall of the pharynx was only slightly stained, if at all.

Nirenstein (1905) maintains that the cytoplasm exerts suction ("eine Art Saugwirkung") on the esophageal sac, and that this causes the sac to enlarge very rapidly to its maximum size and fill with fluid containing but few particles, and he thinks that the cilia in the pharynx then force more particles into the sac and that this causes the observed increase in their concentration. That is, he seems to hold that the cilia come in direct contact with the particles and force them through the fluid in the pharynx and finally into the sac.

Bütschli (1889), Bozler (1924) and others maintain that the enlargement of the esophageal sac is due to pressure of fluid forced into it by the action of the pharyngeal cilia. They consequently do not agree with Nirenstein in reference to the cause of the enlargement, but they appear to agree with him in reference to the process involved in the concentration of particles. Bozler describes this as follows: A drop consisting largely of water ("ein Wassertropfen") is first formed; then solid particles are forced into it by the cilia in the pharynx ("hereingestrudelt") and whirled about by those which project from the end of the pharynx into the drop. After the drop has become nearly filled with particles these cilia rotate its content and press the particles together so as to get as many in as possible.

All the investigators referred to obviously hold that when the esophageal sac begins to enlarge almost nothing but water enters, that this continues until it has nearly, if not entirely, reached maximum size and that after this almost nothing but solid particles enter. This would account for the observed increase in the concentration of solid particles in the esophageal sac, but it is difficult to understand why the cilia in the pharynx should at first transport mainly water and later mainly solid particles.

Lund (1933) agrees with Bütschli and Bozler in reference to the cause of the enlargement of the esophageal sac, but he thinks that "paraesophageal fibrils" at the mouth of the sac are involved in the concentration of particles in it. He says (1933, p. 54): "Food material collects as it reaches these fibrils, so it is probable that they too aid in the concentration of food particles into the vacuoles" and (1941, p. 564) "Here particles of all sizes are trapped in the paraesophageal fibrils, while most of the fluid material presumably circulates back into the pharyngeal cavity. One by one the particles of food slip through the paraesophageal fibrils, and arrive in a growing bulge (the future food vacuole) on the dorsal side of the pharynx."

Lund found in section of paramecia, some particles which appeared to have been entangled in fibrils at the mouth of the esophageal sac. This led to the conclusions quoted above. I have, however, as previously stated, been unable to see in living paramecia any indication of an aggregation of particles at the mouth of the sac or cessation of movement of individual particles in this region. Nor have I been able to find any indication of backward circulation of fluid in the pharynx. The

particles observed by Lund among fibrils at or near the mouth of the sac may well have been deposited there by the microtome knife in sectioning the organisms.

It seems to me to be perfectly obvious that the cilia in the pharynx force fluid with particles in suspension into the esophageal sac and that it is this that causes the enlargement of the sac as Bütschli, Bozler and others maintain. But I could not observe any change in the concentration of the particles in the fluid forced into the sac, during its enlargement, i.e., I did not obtain any evidence indicating that the concentration was at first low and later high as Bozler's view demands. What then causes the observed increase in concentration?

Discussion

Nirenstein (1905) maintains that after the food-vacuole has been formed and has left the pharynx it rapidly decreases in size and that this is due to loss of fluid. These contentions have been abundantly confirmed. The membrane around the food-vacuole is therefore pervious to water, and the loss of water, which causes the vacuole to decrease in size, is doubtless due to excessive external osmotic concentration and inward pressure of the stretched vacuolar membrane (Mast and Bowen, 1944).

If this is true, water must be continuously forced out of the esophageal sac during its enlargement, i.e., during the formation of the food-vacuole, for the factors involved in the loss of water from the fully formed food-vacuole function continuously during its formation. And since no solid particles leave during this time, it is obvious that this would result in increase in their concentration in the forming vacuole. But it does not account for the apparent observed increase in the rate of their concentration as the sac enlarges.

The difference between internal and external osmotic concentration is doubtless practically constant during the enlargement of the esophageal sac, as is also the pressure of the fluid against its inner surface, caused by the action of the cilia in the pharynx. The rate of outflow of water *per unit area* of membrane at the surface of the sac (provided there is no change in its permeability) is therefore practically constant; but since this area increases as the sac increases in size the rate at which water leaves the sac, i.e., the volume per second, also increases. Therefore, since as the sac increases in size the rate of inflow of fluid and particles through the pharynx remains practically constant, while the rate of outflow of fluid without particles increases, the rate of concentration of particles in the fluid in the esophageal sac must increase as the sac enlarges. Moreover, as the esophageal sac enlarges the membrane at its surface is continuously stretched, and as it is stretched it must be continuously built up by the interaction between substances in the sac and the adjoining cytoplasm so as to prevent rupture. It may well be, however, that the membrane thus formed becomes more and more pervious to water as it is stretched during the enlargement of the sac. If this is true, it also causes an increase in the rate of concentration of particles in the sac.

The conclusion that water continuously passes from the esophageal sac into the cytoplasm has an important bearing on various views concerning the origin of the water which is excreted by the contractile vacuoles. Eisenberg (1925) and Frisch (1937) hold that all this water enters the body through the pharynx; Bozler (1924), Fortner (1926), and Müller (1932) conclude that some of it enters through the

pellicle, and Kitching (1934, 1936, 1938) assumes, in some of his experiments, that all enters through the pellicle.

It has been demonstrated by several investigators that the total volume of the food-vacuoles (at maximum size) formed in a given period of time is only about one-third as great as the total volume of the contractile vacuoles (at maximum size) formed during the same length of time. This seems to prove that water enters the body through the pellicle as well as through the pharynx. Eisenberg, Frisch and others maintain, however, that some of the water that enters the pharynx passes directly into the cytoplasm, i.e., without entering the food-vacuole at all, and that this accounts for the observed difference between the total volume of the food-vacuoles and that of the contractile vacuoles. The evidence presented above that water continuously passes from the esophageal sac into the cytoplasm during the formation of the food-vacuoles, strongly supports the contention that the water excreted by the contractile vacuoles enters the pharynx; for the formation of the food-vacuole requires sufficient time to permit, during its formation, the passage of more water from it into the cytoplasm than passes from the food-vacuole into the cytoplasm after the vacuole has been fully formed.

In view of these and other considerations it is difficult to understand how Kitching, in the experiments referred to above, came to assume that all the water that was excreted by the contractile vacuole entered the body through the pellicle.

The Separation of the Food-Vacuole from the Pharnyx and its Movement through the Body

There has been for many years much speculation concerning the processes involved in the separation of the food-vacuole from the pharynx and its movements after it has become free, but these problems still remain unsolved.

I have closely observed under the oil-immersion objective, numerous food-vacuoles leave the pharynx and pass through the body in each of the four species of *Paramecium* listed above, especially the first three. The specimens studied were under many different environmental conditions, both natural and artificial. Extensive variations were seen in each species but the results as a whole seem to show that the processes involved in the closing of the esophageal sac, the separation from the pharynx of the food-vacuole thus formed, and its passage through the body are fundamentally identical in the four species.

The following usually occurs: The esophageal sac enlarges and becomes nearly spherical with the major axis directed backward. The substance in the sac rotates strongly and the particles in suspension vibrate vigorously and become much concentrated. After the sac has become about twice as wide as the pharynx, it slides posteriorly from the diagonal end of the pharynx and becomes definitely pear-shaped, a nipple being drawn out on the vacuole as it leaves the pharynx. (No change whatever in the size of the end of the pharynx can be seen during this process.) A small portion of the esophageal sac remains as a membrane over the opening in the pharynx. This bulges out into the cytoplasm slightly, forming a shallow sac which soon enlarges to form a new vacuole (Fig. 3). After the nipple which connects the newly formed food-vacuole with the tip of the diagonal end of the pharynx has broken, the vacuole moves rapidly *through* the cytoplasm nearly to the posterior end of the body, and on the way it creates marked currents in the

cytoplasm, turns through approximately 270° and becomes spherical. After it has reached the end of this course, it stops a few moments, then slowly passes forward near the aboral surface to the anterior end of the body or part way, and then back along the opposite surface, to the anus.

As stated above, there is great variation in these phenomena. Some of these are set forth in the following paragraph:

During the separation of the food-vacuole from the pharynx, the cytoplasm around it ordinarily flows slowly backward and appears to elongate the vacuole slightly, but sometimes this cytoplasm does not flow at all and occasionally it flows so violently that it appears to elongate the vacuole greatly and actually pull it from

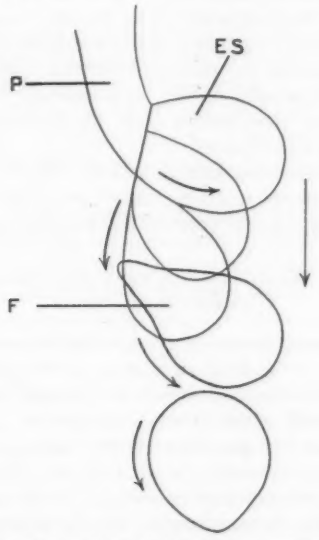

FIGURE 3. Camera outline of a portion of the feeding apparatus and some sketches illustrating the movement of the food-vacuole during its separation from the pharynx in *Paramecium aurelia*. *P*, pharynx; *ES*, esophageal sac; *F*, food-vacuole; arrows, direction of movement.

the pharynx. After the vacuole has been separated from the pharynx it almost invariably moves toward the posterior end of the body much faster than the surrounding cytoplasm, but occasionally it moves so slowly that it appears to be carried by the cytoplasm. On its way toward the posterior end of the body the vacuole usually turns somewhat more than half-way around but sometimes it does not turn at all, and sometimes it turns entirely around and occasionally even more than once; and it sometimes goes only a short distance on its usual course toward the posterior end of the body and is then carried forward. The vacuole usually becomes spherical before it has reached the posterior end of the body, but it sometimes does not become spherical until much later, if at all. This appears to be closely correlated with the viscosity of its content. Details concerning these phenomena are given in the next section (see also page 55–57).

Discussion

It is held by all who have investigated the subject that after the food-vacuole has reached the posterior end of the body, it is carried over the rest of its course in the cytoplasmic stream, i.e., by cyclosis, but there is marked diversity of opinion concerning the processes involved in the separation of the food-vacuole from the pharynx and its rapid movement toward the posterior end of the body.

Stein (1859), Bütschli (1889) and Bragg (1935, 1936) conclude that contraction of the distal end of the pharynx and cyclosis are involved. However, since no decrease in the size of the pharynx can be seen and the vacuole sometimes leaves the pharynx and moves away in the total absence of cyclosis, neither of these two factors is essential.

Nirenstein (1905) maintains that the cytoplasm adjoining the esophageal sac near the distal end of the pharynx contracts and separates the content of the sac from that in the pharynx and that the vacuole thus formed is carried away by cyclosis. Bozler (1924) supports Nirenstein in reference to the separation of the vacuoles from the pharynx, but he holds that the vacuole is not carried off by cyclosis. He contends that there is periodic backward streaming of the cytoplasm (which is not cyclosis) adjoining a group of fibers ("Schlundfaden") which extend from the pharynx nearly to the posterior end of the body and that the vacuole is carried posteriorly in this stream.

If the constriction in the esophageal sac is caused by the adjoining cytoplasm, one would expect to find a differentiated structure in the cytoplasm, i.e., a sort of sphincter. Nothing of this sort has, however, been found; and Bozler's contention that there is a stream of cytoplasm backward along one side of a bundle of fibers has not been confirmed. It is consequently not likely that the formation and movement of the vacuole is dependent upon either of these two postulated factors.

Kalmus (1931) and others seem to think that surface tension plays a predominant role in the formation of food-vacuoles, somewhat as it does in the formation of drops of water in the air. The formation of drops of water in air by the action of surface tension is, however, dependent upon weight. But since food-vacuoles suspended in cytoplasm have no weight, surface tension cannot be essential in their formation.

Gelei (1934) says the food-vacuoles are torn from the pharynx, but he does not say by what means.

Lund (1941) concludes that the food-vacuole is separated from the pharynx and forced toward the posterior end of the body by the action of the "postesophageal fibrils". He says (p. 564): "With the growth of the vacuole the postesophageal fibrils contract about its base, the vacuole is pressed posteriorly, and once it is released the fibrils conduct it into the cytoplasm with considerable rapidity. Their concerted action produces an effect somewhat resembling that produced by a peristaltic wave in the esophagus of higher vertebrates."

Lund did not actually see the processes described nor could I see anything of the sort. I believe, however, that his postulations are eminently sound, for they reasonably account for nearly all that has been seen. There is, however, one observation that seems to require further elucidation.

Nirenstein (1905, p. 451) maintains that if the food-vacuole is directly over or under the diagonal opening of the pharynx which now appears as a clear oval

("heller Oval"), it can be seen that as the vacuole leaves the pharynx, the minor axis of the oval decreases until the oval becomes a slit ("ein heller Spalt") before the major axis decreases. I could not see these phenomena, but the observations are so difficult that this casts no reflections on their validity. Nirenstein thinks that the decrease in the minor axis, before the major axis decreases, must be due to contraction of the cytoplasm ("Plasma") adjoining the esophageal sac near its connection with the pharynx. Lund asserts, as previously stated, that there are several fibers which extend over the opening at the distal end of the pharynx, i.e., the mouth of the esophageal sac. I have already presented evidence which shows that if these fibers exist (and Lund's figures seem to prove that they do), they are not inside of the pharynx or the sac. They must, therefore, be outside, some doubtless being on either side of the sac close to the end of the pharynx. If this is true, these two groups of fibers become much separated in the middle and stretched as the sac enlarges. And if they now contract the opening of the sac will be laterally squeezed and consequently will become slit-like, i.e., it will assume a form which is in accord with Nirenstein's contention. It may well be, therefore, that these fibers (called "paraesophageal fibrils" by Lund) function in the separation of the food-vacuole from the pharynx.

Initiation of the Separation of the Food-Vacuole from the Pharynx

The earlier investigators held that in *Paramecium* the size of the food-vacuole controls the initiation of its separation from the pharynx, and this idea seems still to be widely held. For example, Bragg (1935) says the food-vacuole "grows gradually larger till, reaching its full size, it suddenly drops into the endoplasm." This contention is supported by the fact that in a given individual successive vacuoles are usually nearly the same in size, but it does not account for the great difference in their size often observed.

Bozler (1924) asserts that the separation of the food-vacuole from the pharynx is initiated by contact of particles with the inner surface of the vacuolar membrane. He bases this assertion on his contention that paramecia will not form food-vacuoles in a medium which does not contain particles. In this contention he does not, however, agree with Schewiakoff (1894), who maintains that particles are not necessary. Bragg (1935) says that in *Paramecium trichium* contact of large particles with the inner surface of the vacuolar membrane is always immediately followed by separation of the vacuole from the pharynx. This seems to support Bozler's contention. Bragg says however that many of the food-vacuoles formed in this species have no large particles and that in *Paramecium caudatum* large particles in the vacuole have no effect on their separation from the pharynx. He consequently concludes that they are not necessary.

I made many observations concerning this on each of the four species of *Paramecium* under consideration and found in all these species that the food-vacuole frequently leaves the pharynx immediately after a large particle has entered but that this does not always occur in any of them. I observed that when *Paramecium trichium* is ingesting yeast-cells, the food-vacuole usually leaves the pharynx immediately after one of these cells has entered but that occasionally it does not leave until after two have entered. I also found that this obtains for each of the three other species studied, when they are ingesting specimens of *Chilomonas paramecium,* and in these

I observed that occasionally the vacuole did not leave the pharynx until after three had entered.

These results are therefore not in full accord with Bragg's contentions. However, I also observed in all the species, food-vacuoles which had no large particles whatever. This supports Bragg's conclusion that contact of large particles with the vacuolar membrane is not necessary for the separation of the vacuole from the pharynx. This conclusion is also supported by the fact that I have under various conditions seen many food-vacuoles form in which no particles whatever could be seen. It is therefore very probable that in Bozler's observations the failure of the paramecia to form food-vacuoles in a particle-free medium was due to injurious action of the medium he used rather than to the absence of particles in it.

The evidence in hand obviously throws but little light on the processes involved in the initiation of the separation of the food-vacuole from the pharynx in paramecia, and this is also true for other forms. Mast and Bowen (1944) considered this problem theoretically in reference to the Peritricha and reached the following conclusions: "It is highly probable that waves start at fairly definite intervals in the pharyngeal ring and pass simultaneously down all the esophageal fibers and that each of these sets of waves initiates a constriction in the esophageal sac, if it contains sufficient fluid to make a constriction possible. If this is true, the size of the vacuole is correlated with the rate at which the fluid is forced into the esophageal sac by the cilia in the pharynx and the rate at which it leaves this sac by osmosis. If these processes and the interval between successive waves depend upon the composition of the surrounding fluid, the temperature and the physiological state of the organism, it accounts for the observed variation in the size of the food-vacuoles and the intervals between their formation." It seems to me that these conclusions apply equally well to *Paramecium*. Moreover, Bozler (1924) says that in this genus a momentary current passes posteriorly along the "Schlundfaden" at about 50-second intervals. These currents, he asserts, are entirely independent of cyclosis. I have seen similar currents in this region, though not very distinctly. I believe they are produced by periodic action of the esophageal fibers. If this is true, it supports the conclusions presented above.

Size, Rate of Formation, and Shape of the Food-Vacuole

Size

It is well known that the food-vacuoles formed in *Paramecium* vary greatly in size, but very little is known concerning the cause of this variation.

Metalnikow (1912) maintains that if paramecia are transferred from a solution which is poor to one which is rich in digestible substance (i.e., bacteria, milk, egg-yolk, etc.) the first vacuole formed is always huge, judging from his figures, nearly 40 times larger than normal vacuoles. He also maintains that in solutions which contain only indigestible particles (carmine, chinese ink, etc.) the food-vacuoles formed are abnormally small. He does not designate the species studied, but his figures indicate that it probably was *aurelia*.

I repeated Metalnikow's experiments and obtained results which on the whole support his contention; but I found that the size of the vacuoles is not at all closely correlated with the composition of the surrounding medium. That is, I found that

the extent of change in the size of the vacuoles formed after transfer to a given medium varied greatly.

Dogiel et al. (1927) made observations on the food-vacuoles formed by paramecia [4] in solutions of salts, containing numerous particles of chinese ink in suspension. They found that in solutions of $MgCl_2$, $MgSO_4$, and $FeSO_4$, the food-vacuoles formed are long, tubular and coiled ("Nahrungsschlauche") but that in solutions of $BaCl_2$ they are minute and spindle-shaped. Judging from the sketches presented, the former would average about 60 μ in diameter in spherical form, and the latter only about 4 μ. Both were filled with particles of ink.

I repeated these experiments on each of the four species of *Paramecium* listed above, with each of four brands of chinese ink in a much greater range of concentrations of the salts than was used by Dogiel et al. Moreover, the solutions of all the salts were made up respectively in distilled water, tap-water, and fluid taken from the cultures. The paramecia ingested the particles of ink freely in all the salt concentrations tested, except those which were so high that they were injurious; but I observed no indication of the formation of abnormal food-vacuoles in any of the solutions, either in form or in size.

Dogiel et al. assume that the abnormal food-vacuoles they observed were due to the action of the salts used. They say, however, that they did not obtain such vacuoles if the chinese ink was omitted. This, and the fact that I obtained no abnormal vacuoles, strongly indicates that the abnormality observed by them was due to the action of the ink they used rather than the salts.

Cosmovici (1931) maintains that *"Colpidium colpoda"* in culture fluid containing "amylodextrine" forms tubular food-vacuoles, some of which extend from the pharynx to the anus. He concludes that there is in the ciliates a very complicated closed capillary digestive system, through which substance is moved by waves of cytoplasmic contraction, and that cyclosis is an optical illusion, due to this movement. Amazing conclusions!

I repeated and extended Cosmovici's observations using paramecia in place of colpidia, but obtained no evidence whatever in support of his contentions.

Frisch (1937) made very extensive measurements on food-vacuoles in specimens of *Paramecium nucleatum* taken from given cultures on successive days for three weeks or more. The cultures remained in a flourishing condition for nearly two weeks and then declined. During the flourishing period the average diameter of the food-vacuoles formed in different individuals varied from 17.25 μ to 25 μ with 65.55 μ for the largest vacuole measured. As the cultures declined the food-vacuoles formed decreased in diameter "first to 13.80 microns, then progressively to 10.35, 6.90 and 3.45 microns." The paramecia were plump and ranged from 227 μ to 330 μ in length until the cultures began to decline, then they gradually became thin and somewhat shorter. It is consequently evident that in these cultures the size of the food-vacuoles formed varied directly with the size of the paramecia. Frisch maintains, however, that in well-fed paramecia the size of the food-vacuoles is not closely, if at all, correlated with the size of the body and that the observed decrease in the size of the food-vacuoles during the decline of the cultures was largely if not entirely, due to decrease in quantity and quality of food, i.e., bacteria.

[4] The species is not designated but the size of the paramecia calculated from the sketches presented and the fact that only one micronucleus is figured, indicate that it was *caudatum*.

I repeated the observations made by Frisch and extended them to other species. I also made observations on the effect of increase in the viscosity of the surrounding fluid. The results obtained are in full accord with those obtained by Frisch. I did find, however, that while within a given species, the size of the food-vacuoles does not appear to be correlated with the size of the individuals, the vacuoles formed by the two smaller species (*aurelia* and *trichium*) were, in general, much smaller than those formed by the two larger (*caudatum* and *nucleatum*) and that the size in all the species appears to depend upon the viscosity of the surrounding medium.

In the observations on the effect of viscosity, polyvinyl alcohol or methyl cellulose was added to culture fluid containing paramecia on a slide. It was found that if the viscosity of the fluid became high enough to retard locomotion, but not high enough to inhibit it, the paramecia ingested the fluid rapidly, formed unusually large food-vacuoles and soon became well filled with them (Fig. 4). In some of these vacuoles a considerable number of solid particles could be seen but in others none

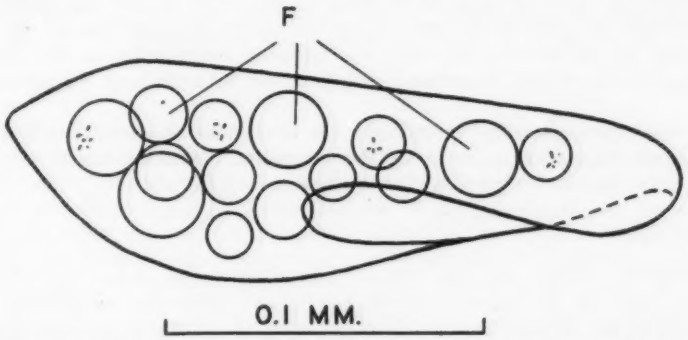

FIGURE 4. Camera outline of *Paramecium caudatum* showing food-vacuoles formed in a viscous solution of polyvinyl alcohol. *F*, food-vacuoles. The small dots in the food-vacuoles represent solid particles. Most of the vacuoles did not contain any.

whatever, despite thorough examination of much flattened specimens under the oil-immersion objective. Bozler (1924) obtained somewhat similar results with other viscous substances.

Lee (1942) asserts that "preliminary observations" show that the size of the food-vacuoles in *Paramecium* is independent of the hydrogen-ion concentration of the surrounding medium, but he gives no details regarding the results of the observations made. It may well be, therefore, that this assertion is not strictly true. However this may be, the evidence in hand seems to show that there are at least four environmental factors which are involved in the control of the size of the food-vacuoles, namely, the quantity and the quality of the particles in suspension, and the chemical composition and the viscosity of the surrounding fluid. The question now arises as to how these factors function.

Mast and Bowen (1944) found in observations on *Vorticella similis* that the food-vacuoles vary *greatly* in size without any variation in the number or kind of particles suspended in the surrounding fluid or in its chemical composition. But

they did find that under certain conditions the size is definitely correlated with the quantitative rate of ingestion.

They postulate, as stated above, that the food-vacuole is formed by constriction in the esophageal sac caused by the action of the esophageal fibers and that this constrictive action occurs at fairly regular intervals regardless of the size of the esophageal sac. If this is true, it is obvious that if the constrictive action occurs when the sac is large, a large vacuole will be formed and that if it occurs when the sac is small, a small vacuole will be formed; and it is also obvious that if the quantitative rate of ingestion is high the esophageal sac will become, during the interval between successive constrictive actions, larger than if it is low. But the size of the sac will also depend upon the quantitative rate of passage of fluid from the sac through its surface membrane into the adjoining cytoplasm. According to this view then the primary factors involved in controlling the size of the food-vacuoles are (1) the quantitative rate of ingestion of fluid and solid particles, (2) the quantitative rate of passage of fluid from the esophageal sac into the cytoplasm, and (3) the length of the intervals between consecutive constrictive actions of the esophageal fibers. If this obtains all other factors that may be involved (the quantity and the quality of particles in the surrounding fluid, the chemical composition of this fluid, the physiological state of the organisms, etc.) function by modifying the primary factors.

This hypothesis is in full accord with the facts in hand, concerning the control of the size of the food-vacuoles in the Peritricha, and it also appears to account for all that is known concerning this phenomenon in *Paramecium*.

Rate of formation

Metalnikow (1912) contends that the rate of formation of food-vacuoles is dependent upon the hydrogen-ion concentration and upon the temperature of the surrounding fluid but that it is not dependent upon the number of particles suspended in it.

Bozler (1924) asserts that the formation of a food-vacuole requires 4 to 5 minutes if the particles in suspension are scarce and only 1 to 2 minutes if they are abundant. He consequently does not agree with Metalnikow in this respect.

Frisch (1937) seems to agree with Bozler in reference to the correlation between the concentration of particles and the rate of formation of food-vacuoles. He found that the time required for the formation of the food-vacuoles measured in the observations described above, varied from 17 to 365 seconds, and he maintains that the time required for the formation of a food-vacuole is not dependent upon the size of the vacuole but that it is largely, if not entirely, dependent upon the quantity and the quality of the food present. He writes: "This variation did not depend upon the size of the food-vacuole formed but probably upon the quantity and the quality of bacteria present in the immediate vicinity of the animals." He does not elucidate this dependence, but he doubtless holds that the time varies inversely with the quantity of bacteria and the extent of their usefulness as food.

Lee (1942) demonstrated that the rate of formation of food-vacuoles is closely correlated with the hydrogen-ion concentration and the temperature of the surrounding fluid. He consequently supports Metalnikow.

No definite views have been expressed as to how the factors involved act in the

control of the rate of formation of food-vacuoles. Lee implies, however, that the rate is directly proportional to the activity of the cilia in the vestibulum ("peristome") and that this is correlated with acidity, temperature, etc. This would account for the facts observed by Lee, but it would not account for the correlation described above between the rate of formation of vacuoles and the concentration of particles in suspension in the surrounding medium. Moreover, I have repeatedly seen paramecia in which the cilia in the vestibulum were very active and numerous particles entered the vestibulum, but none passed into the pharynx, all being thrown out. This indicates that the amount of substance which enters the pharynx depends upon the nature of the activity of the cilia in the vestibulum quite as much as upon the magnitude of the activity.

If the food-vacuoles are separated from the pharynx by constrictive actions of the esophageal fibers and these actions occur at regular intervals in accord with the hypothesis presented above, the rate of formation of the vacuoles must depend upon the length of these intervals; and the effect on the rate of formation produced by the factors considered above, and others (i.e., acidity, temperature, physiological states, etc.) must be due to alteration produced by them, in the length of the intervals.

Shape

It is generally agreed that the newly formed food-vacuoles in *Paramecium* are usually nearly spherical but that they sometimes are distinctly spindle-shaped (Nirenstein, 1905; Bozler, 1924; Dunihue, 1931; Gelei, 1934; Bragg, 1935, 1936). There is no controversy concerning the cause of the spherical form, but there are several views as to the cause of the spindle-shape.

Nirenstein holds that the spindle-shape is due to constriction of the distal end of the forming vacuole by contraction of the adjoining cytoplasm and to drawing out of the proximal end to a point as the vacuole leaves the pharynx. Bozler maintains that the food-vacuole is pressed against a bundle of fibers ("Schlundfaden") on its way from the pharynx to the posterior end of the body and that this causes the spindle-shape. Gelei found that the portion of the esophageal sac which remains attached to the pharynx after a food-vacuole has left, is sometimes pointed and he maintains that this results in a point on the distal end of the following vacuole and consequently in a spindle-shape. Mast and Bowen (1944) found that this regularly occurs in the Peritricha in which the newly formed food-vacuole is always spindle-shaped. Their results consequently support Gelei's contention. Bragg (1936) concludes that the food-vacuole, immediately after it leaves the pharynx, is usually spindle-shaped in *Paramecium caudatum,* rarely in *P. nucleatum* and never in *P. aurelia* and *P. trichium.* He concludes that its form is a species characteristic, but he gives no information as to why, in a given species, it is sometimes nearly spherical and sometimes definitely spindle-shaped.

I found in observations on paramecia which had been in culture fluid containing neutral red in moderate concentration, that nearly all the food-vacuoles formed in *P. caudatum* and *P. nucleatum,* but none of those formed in *P. aurelia* and *P. trichium,* were spindle-shaped. I also found that, while in the spherical vacuoles there was invariably violent movement, in the spindle-shaped vacuoles there was little or none. This shows that the viscosity of the fluid in the vacuoles differs greatly and that the shape of the vacuoles is correlated with it.

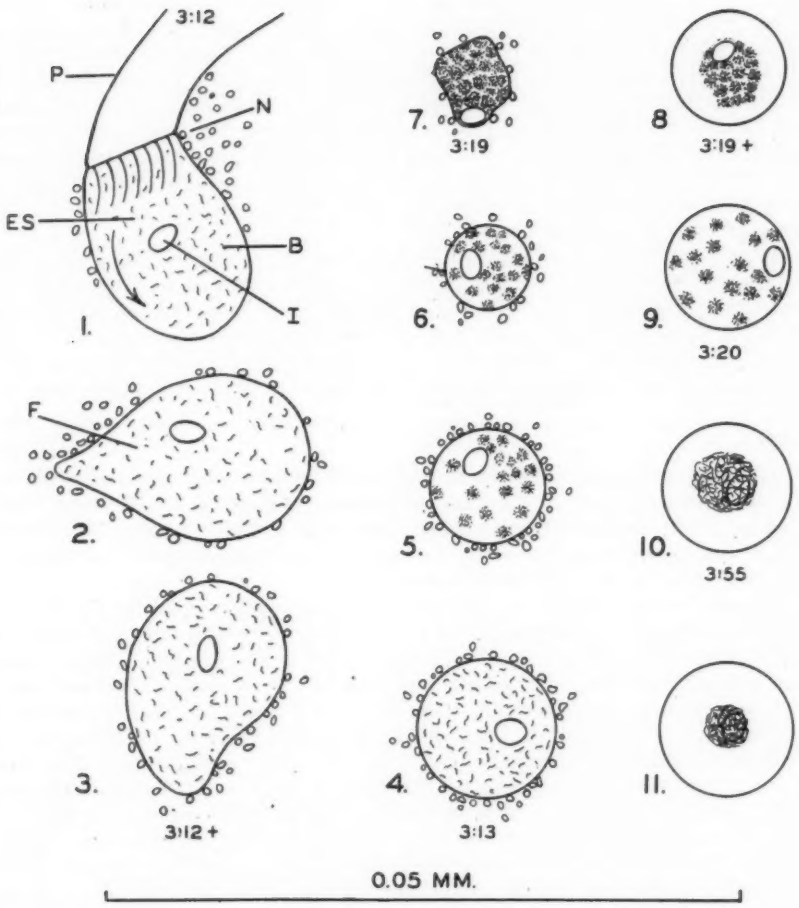

FIGURE 5. Camera outlines of a food-vacuole in a specimen of *Paramecium aurelia*, showing changes in form, size, and content, and changes in the position of neutral-red granules at the surface. *P*, pharynx; *ES*, esophageal sac; *F*, food-vacuole; *B*, bacteria; *I*, indigestible body; *N*, neutral-red granules; 3:12–3:55, time.

Note that the vacuole rapidly became spherical then decreased slowly but greatly in size, then increased rapidly and greatly. Note also that the numerous neutral-red granules at the surface of the vacuole all disappeared during its rapid enlargement and that the bacteria appear to have died and agglutinated during the decrease in size.

Changes in Shape and Size of the Food-Vacuole after it has left the Pharynx

Nirenstein (1905) contends that the food-vacuole in *Paramecium,* after it has left the pharynx, becomes spherical and decreases in size to about one-tenth and then increases until it is somewhat larger than it was originally. These contentions have been confirmed by others.

I made detailed observations concerning these phenomena in *Paramecium caudatum, P. nucleatum,* and *P. aurelia.* The results obtained show that they are fundamentally the same in these three species. The changes observed in a typical food-vacuole in *Paramecium caudatum* are illustrated in Figure 5.

This figure shows that the vacuole, after it had become free from the pharynx, rapidly became spherical and slowly decreased in size to nearly one-twentieth and then very rapidly increased until it was nearly as large as it had been originally. The figure indicates that as the vacuole decreased in size the bacteria in it died and aggregated in numerous small clumps, and that fluid passed out into the surrounding cytoplasm until there was practically none left, and the vacuole was so packed full of solid particles that the surface was very irregular. The figure indicates also that the numerous "neutral-red granules" at the surface of the vacuole when it left the pharynx, disappeared during its rapid enlargement. All these phenomena varied greatly in extent and time. For example, under some conditions there was no perceptible change in the size of the vacuoles at all. Details concerning some of them are given in the next section (see pages 55–57).

Mast and Bowen (1944) observed similar phenomena in the food-vacuoles in the Peritricha. They make the following statements in reference to the causes of the changes in size: "The loss of fluid, resulting in the decrease in the size, is probably due in part to difference in the osmotic concentration of fluids in the vacuoles and the cytoplasm [5] (that of the latter being higher than that of the former) and in part to inward pressure of the elastic membrane on the surface of the vacuoles, which was stretched by the pressure of fluid forced into them by the pharyngeal cilia. The inflow of fluid, resulting in increase in size, is probably entirely due to greater osmotic concentration within the vacuole than without. If this is true, the internal osmotic concentration must increase greatly during the time that the vacuole remains minimum in size. This could readily be brought about by transformation in the vacuole of osmotically inactive to osmotically active substance, for example, starch to sugar. In food-vacuoles which contain lactose, the gelatinous substance in them, referred to above, increases greatly in viscosity as the vacuoles decrease in size (as indicated by observations on Brownian movement) and then decreases greatly as they increase in size. The increase in viscosity is correlated with increase in acidity. It may well be that this increase in acidity causes chemical changes in the gelatinous substance which result in increase in osmotic concentration and that this

[5] This contention is strongly supported by the following observations: Mast and Bowen (1944) found that the food-vacuoles in *Vorticella similis* do not decrease in size if lactose in sufficient quantity is added to the culture fluid; and Fortner (1933) found the same in *Paramecium caudatum* in culture fluid to which *numerous* crushed bacteria had been added. In both of these observations the osmotic concentration of the fluid in the food-vacuoles was probably as high or higher than that of the surrounding cytoplasm, hence the retention of the water in them and no decrease in size.

in turn causes the rapid inflow of fluid from the cytoplasm which in turn, owing to decrease in acidity, causes the observed decrease in viscosity."

These statements are equally applicable to the food-vacuoles in *Paramecium*.

The Hydrogen-ion Concentration of the Content of the Food-Vacuole in Paramecium

It has been known for many years that after the food-vacuole in protozoa has been formed the hydrogen-ion concentration of its content increases considerably and then decreases again (Metchnikoff, 1889; LeDantec, 1890; Greenwood and Saunders, 1894; Nirenstein, 1905; et al.[6]). There is however marked diversity of opinion concerning the magnitude of these changes and the factors involved in producing them. Moreover, it has recently been maintained that in *Paramecium* the ingested substance becomes alkaline before the food-vacuole is formed, i.e., that there is in the vacuole a "preliminary alkaline phase."

The preliminary alkaline phase of the food-vacuole

Shipley and DeGaris (1925) made observations on specimens of *Paramecium caudatum* and *P. aurelia* in acid culture fluid to which phenol-sulfonphthalein (phenol red) had been added. They maintain that this fluid and the bacteria in it were distinctly yellow (acid), but that as it passed through the pharynx it became "faintly but distinctly pink . . . next to the wall of the pharynx", and that the forming food-vacuole was "filled with an alkaline [pink] fluid" which later became yellow (acid), and then pink (alkaline) again. They seem to hold that an alkaline substance is secreted by the wall of the pharynx.

Shapiro (1927) repeated the observations of Shipley and DeGaris and extended them to paramecia in neutral and in alkaline culture fluids. He says that in the neutral culture fluid "the vacuoles at first had a very light shade of pink" (alkaline), but that in the acid and the alkaline culture fluids there was no evidence of this. He concludes, however (p. 49), that "the observations made by Shipley and DeGaris of an alkaline stage at the beginning of the cycle has been verified." Dunihue (1931) asserts, on the contrary, that he was unable to confirm these observations.

I put into each of seven test-tubes a given quantity of fluid from a vigorous culture of *Paramecium caudatum* and added a different quantity of phenol red to the fluid in each, so as to produce a graded series of concentrations. All the solutions became distinctly yellow, and comparison with a series of buffers containing phenol red, showed that they were acid. After 24 hours the paramecia in the two strongest solutions were dead, but those in the rest of the solutions appeared to be in excellent condition. Some from each of these solutions were mounted and studied. They ingested fluid and bacteria in all, and the results obtained were the same in all.

[6] Engelmann (1879, p. 349) had previously observed ingested blue particles of litmus become red in *Paramecium aurelia, Stylonychia mytilus,* and *Amoeba diffluens.* But he did not know that this change in color was correlated with the content of the food-vacuoles. He assumed that the ingested particles of litmus were in direct contact with the cytoplasm and he therefore concluded that the observed change in their color demonstrated that the protoplasm is acid in these species.

Under a magnification of 400 (20 oculars and 20 objective) the stream of fluid in the pharynx was definitely yellow except a thin layer at the surface, i.e., a thin layer adjoining the wall of the pharynx, which was distinctly purplish pink. The fluid at the surface of the forming food-vacuole was also purplish pink, but that in the central portion was yellow, and the bacteria in it appeared to be the same in color as the fluid. After the vacuoles left the pharynx, they decreased slowly in size and the bacteria became intensely yellow, much deeper yellow than the fluid around them. Later the vacuoles increased rapidly in size, and the fluid in them became light purplish pink and the bacteria dark purplish pink.

These results are essentially in accord with the contentions of Shipley and DeGaris, and they appear to support their implication that the wall of the pharynx secretes an alkaline substance. However, on further examination of the paramecia in the culture fluids containing phenol red, I found that the surface of the content of the contractile vacuoles was also purplish pink, that is, that it was the same in color as the surface of the content of the pharynx, and I later observed the same purplish pink color in the pharynx and the contractile vacuoles in paramecia in culture fluid which did not contain any dye. Moreover, under a magnification of 1200 (20 oculars and 60 oil-immersion objective) there was no indication whatever of purplish or pinkish color in the pharynx or the contractile vacuoles, either in the paramecia in the culture fluid containing phenol red or in those in the culture fluid without any dye.

These results seem therefore to indicate that the purplish pink color observed in the lower magnification was due to refraction phenomena, not to change in acidity. It is consequently highly probable that the pinkish color observed in the pharynx and the forming food-vacuole by Shipley and DeGaris and Shapiro was not due to decrease in acidity.

I made numerous observations with several other indicator dyes, concerning a decrease in the acidity of the fluid in the pharynx in *Paramecium* but obtained no evidence of any. Moreover, Lund (1914) demonstrated fairly conclusively that in *Bursaria* there is a definite increase. That is, he found precisely the opposite from what Shipley and DeGaris maintain occurs in *Paramecium*. The evidence in support of a "preliminary alkaline phase" in the food-vacuoles in *Paramecium* is consequently negligible.

The Magnitude of the Changes in the Hydrogen-ion Concentration in the Food-Vacuole

Bozler (1924) was probably the first to attempt to measure the hydrogen-ion concentration of the content of the food-vacuole. He made observations on food-vacuoles in paramecia which had ingested yeast-cells stained with congo red; and he concluded that the hydrogen-ion concentration of their content increases approximately to pH 3. Nirenstein (1925) repeated and extended Bozler's observations and concluded that it increases nearly, if not quite, to pH 1. Neither of these investigators measured the subsequent decrease in hydrogen-ion concentration. Shapiro (1927) measured this and also the preceding changes. He made observations on paramecia in culture fluid containing respectively litmus, congo red, and phenol red, and concluded that in neutral culture fluid the acidity at first decreases to about pH 7.6 ("preliminary alkaline phase"), then increases to a maximum of

pH 4, and then decreases about to pH 7. Kalmus (1931) confirmed Nirenstein's conclusion.

I repeated and extended the preceding observations made with dyes and I also made observations on ingested crystals.

Ingested crystals

Neutral-red crystals were produced and tested for solubility and changes in color in relation to hydrogen-ion concentration as described in preceding papers (Mast, 1942; Mast and Bowen, 1944). Some fragments of these were added respectively to neutral and slightly acid or alkaline culture fluid containing paramecia on a slide and covered with a cover-glass supported on ridges of vaseline.

In some tests the culture fluid was taken from vigorous cultures which contained numerous bacteria; in others it was taken from old cultures which contained only a few bacteria, and in still others it was taken from a fresh culture which contained very few bacteria. Starch was added to the culture fluid in some of the tests. Observations were made on three different species of paramecia, namely, *caudatum*, *nucleatum*, and *aurelia*. The results obtained are essentially the same for the three species studied.

The paramecia fed freely in all the solutions used. Some of the food-vacuoles formed contained, in addition to the culture fluid ingested, only bacteria, others chiefly starch, and still others fragments of crystals, bacteria, and starch in various proportions. The fragments of crystals ingested ranged in size from irregular masses not much larger than the bacteria, to needle-like structures nearly half as long as the diameter of the paramecia.

The largest fragments invariably punctured the wall of the vacuole and passed out into the cytoplasm. Some of these were continuously observed under the oil-immersion objective for an hour or more. During this time they circulated several times around the body in the cytoplasmic stream and were then ejected. A few "neutral-red granules" became attached to the surface but no perceptible change occurred in the crystals, either in color or in form.

All the smaller fragments of crystals ingested remained in the food-vacuoles. These in most of the vacuoles did not change perceptibly and were ejected intact with the rest of the undigested substance. But those in some changed in color from brownish yellow to pink, and some of these rounded up and became nearly spherical. I did not see any disintegrate completely; but the fact that they rounded up shows that they became plastic and that they probably had partially dissolved.

These changes invariably occurred soon after the vacuoles had left the pharynx, but not until after they had decreased considerably in size. They occurred in vacuoles which contained only a few bacteria as well as in those which contained many, but not in all, and they did not occur in any vacuoles which were well filled with starch grains and contained but little fluid, and consequently decreased but little in size.

In acetate buffer solutions the crystals became pink immediately at pH 5 and lower, and dissolved in a few seconds. At pH 5.6 they became pink at the surface in 2 to 3 minutes and dissolved in 15 to 60 minutes. In hydrochloric acid, pH 4, they became pink at the surface immediately and dissolved in 10 to 20 minutes, but at pH 5 it required several minutes for them to become pink and more than 60

minutes for all to dissolve. Neutral-red crystals appear therefore to be considerably more readily soluble in acetate buffer solutions than in solutions of hydrochloric acid in distilled water. This difference seems to be correlated with the difference in the amount of buffers present.

The fact that the crystals became pink and, at least partially, dissolved in a few minutes in some food-vacuoles and did not change perceptibly in others shows that the maximum hydrogen-ion concentration of the content of the vacuoles varies considerably. This variation seems to be, at least in part, due to difference in the extent of the decrease in size of the vacuoles, for there was, as stated above, no change in the crystals in any of the vacuoles until after the vacuoles had decreased considerably in size.

The fluid in the food-vacuoles is doubtless well buffered and consequently resembles acetate buffer solution in this respect. Therefore, the facts that in acetate buffer solution the crystals become pink at pH 5.6 and that in many of the food-vacuoles they do not become pink indicate that the hydrogen-ion concentration of the fluid in these vacuoles does not reach pH 5.6. The results presented below show, however, that the maximum acidity reached in other vacuoles is very much higher.

Ingested dyes

The observations on ingested dyes were made as follows: A series of small test-tubes, containing some of the dye under consideration and appropriate buffer solution which differed by pH 0.2 in successive tubes, was prepared. Then culture fluid containing paramecia and bacteria was mounted between two parallel ridges of vaseline on a slide and dye or yeast-cells which had been stained by boiling in water containing dye, added. Then the preparation was covered with a cover-glass and the color of the content of the food-vacuoles formed, compared with that of the buffer in each of the test-tubes, and usually also with that of stained yeast-cells mounted respectively in buffer solution from each of the test-tubes. The hydrogen-ion concentration of the content of the food-vacuole was assumed to be that of the buffer and the stained yeast-cells in it, which it most nearly resembled.

Neutral red (pH 6.8, red—pH 8, auburn)

Three species of *Paramecium* were studied, *caudatum*, *nucleatum*, and *aurelia*. The results obtained were essentially the same in these three species.

Owing to the activity of the paramecia, it was usually impossible to keep a given food-vacuole under observation for more than a few minutes, especially under high magnification. I succeeded however in continuously observing one vacuole under 20 oculars and a 60 oil-immersion objective throughout practically its entire existence. Since these observations are exceptional and the results obtained concern various important phenomena aside from changes in hydrogen-ion concentration, they will be presented in detail.

The paramecium in which this vacuole formed, had been for 24 hours in culture fluid containing numerous bacteria and neutral red in low concentration. When it was discovered there were scattered through the cytoplasm about a dozen food-vacuoles and numerous small red granules or droplets often called "neutral-red granules." The vacuoles varied greatly in size and color. Some were pinkish red,

others yellow and still others various shades between. The red granules were much more abundant in the region around the distal end of the pharynx than elsewhere. They were especially abundant near the distal end of the aboral surface of the pharynx and the surface of the forming food-vacuole. There were so many granules on these surfaces that they appeared to form a continuous layer. Some of the formed food-vacuoles were also well covered with granules but others had none at all.

The food-vacuole under observation began to form about 3:11 P.M. It contained numerous bacteria and a small yellow neutral-red crystal which served admirably to distinguish the vacuole from others in the body. The bacteria were swimming actively and the entire content of the vacuole rotated rapidly, owing to the action of the cilia which projected from the pharynx into it. The vacuole left the pharynx at 3:12 P.M. and passed very rapidly nearly to the posterior end of the body. It turned approximately through 180° on the way, created violent currents in the cytoplasm and dragged numerous neutral-red granules after it. It was pear-shaped when it left the pharynx but became nearly spherical on the way to the posterior end of the body, i.e., in about one-fourth second. The bacteria and the fluid in it now appeared to be distinctly pinkish in color, but this was due to the red granules on the surface, for careful focusing showed that both the fluid and the bacteria in it were colorless. By 3:12½ P.M. the vacuole had decreased about one-fifth in diameter and the crystal had become spherical and crimson in color, but the bacteria were still colorless and active and the fluid was also still colorless. The crystal had apparently partially dissolved and had become plastic. At 3:15 P.M. the bacteria were motionless and faintly pink in color, but the fluid was still colorless. No further change had occurred in the crystal and the surface of the vacuole was still well covered with neutral-red granules. Some of these granules were seen to leave and others to approach the surface. *None passed into the vacuole.* By 3:17 P.M. the vacuole had decreased nearly one-half in diameter and had moved forward considerably. The bacteria had become deep pink in color, but no perceptible change had occurred in the fluid or the crystal or in the number of neutral-red granules on the surface. At 3:19 P.M. the diameter of the vacuole was only about one-fourth its original length. The bacteria were closely packed around the crimson crystal, forming a dark pink mass closely surrounded by the vacuolar membrane which was still well covered with red granules. A few moments later the vacuole began to increase rapidly in size. At 3:20 P.M. it had nearly reached its original size. During its enlargement the bacteria had become lighter in color, the fluid in the vacuole remained colorless and the neutral-red granules on the surface disappeared. By 3:23 P.M. the bacteria had become colorless, considerably larger and indefinite in outline and the crystal had become nearly colorless. No further changes in color occurred in the content of this vacuole. None of it became yellow. This was doubtless due to excessive dilution of the dye during the enlargement of the vacuole as indicated below. These results were confirmed by observations for shorter periods on many other food-vacuoles in this preparation.

In observations on food-vacuoles in paramecia in other preparations, some of which contained stained yeast-cells, the following was found: Under some conditions the content of the forming vacuole did not rotate, the bacteria were quiet and there was no Brownian movement. This indicates that the fluid in it was very viscous. The newly formed food-vacuoles were usually well covered with neutral-red gran-

ules, but occasionally there were only a few on the surface. Sometimes all the granules left the surface before the vacuole had decreased to minimum in size, but there was no indication that any entered the vacuoles. In preparations which contained but little neutral red, the neutral-red granules did not stain, but the bacteria in the food-vacuoles still became pink after they died. All but a few of the food-vacuoles observed were nearly spherical when they left the pharynx, and all these rapidly became spherical after they had left. The rest were spindle-shaped when they left the pharynx. Most of these rounded up very slowly after they had left and a few of them probably did not become spherical at all. Nearly all the spindle-shaped vacuoles observed were formed in paramecia which had been subjected for twelve hours or longer to relatively strong solutions of neutral red.

In most of the food-vacuoles observed a yellow hyaline layer formed at the surface, as the vacuoles enlarged after having decreased to minimum in size. This layer surrounded a red mass consisting of bacteria and other particles which gradually became uniformly distributed and distinctly yellowish in color. In the rest of the vacuoles observed, no layer formed at the surface, the distribution of the bacteria and other particles keeping step with the enlargement of the vacuoles; but the entire content of these vacuoles eventually became yellow, just as it did in those in which a layer had formed.

Ordinarily the food-vacuoles, after they had enlarged, remained intact until their undigested content was eliminated; but in a few instances two or more were seen to fuse, and some of the vacuoles thus formed were very large.

The results presents show that there is great variation in all the phenomena observed in the food-vacuole, but that the change in the acidity of its content is closely correlated with change in its size. They indicate that, as the vacuole decreases in size, the hydrogen-ion concentration of its content increases in some instances to more than pH 4 and then (as it increases in size) decreases approximately to pH 8. The colors of the neutral-red-containing buffers used in measuring the acidity of the neutral red stained content of the food-vacuoles, were however so indefinitely correlated with the acidity of the buffers that the results obtained are necessarily little more than crude approximations.

The Maximum Acidity of the Content of the Food-Vacuole

Congo red (pH 3, blue—pH 5, orange)

As stated above, congo red has been used by several investigators to measure the maximum acidity of the content of the food-vacuole in *Paramecium* and the conclusions reached differ greatly. In attempting to elucidate this diversity I extended the observations which have been made, using *Paramecium caudatum, P. nucleatum, P. aurelia,* and *P. trichium.*

In specimens of *P. caudatum* mounted in culture fluid containing numerous bacteria and congo red in excess, the following was found: The fluid in the newly formed food-vacuoles was light yellow, the undissolved particles of congo red, yellowish brown and the bacteria, colorless. As the vacuoles decreased in size after they had left the pharynx, the fluid in them decreased greatly in quantity and became light blue, the bacteria died and became dark blue and the particles of congo red became dark blue; then as the vacuoles increased in size, the fluid in them increased greatly

in quantity and became orange, after which the bacteria and the particles of congo red soon also became orange, and remained so until they were eliminated.

In some of these observations the paramecia, after having been a few minutes in the culture fluid containing bacteria and congo red, were transferred with as little fluid as possible, to a smear of polyvinyl alcohol on a slide and covered with a coverglass. These paramecia became so quiet that they could readily be studied under high magnification. Some of them contained food-vacuoles in which all the changes described above were very distinctly seen. When these food-vacuoles had become minimum in size the color of their content resembled that of buffer pH 3.2. These results therefore indicate that the maximum acidity of the content of the food-vacuoles in *Paramecium* is approximately pH 3.2 and that this is reached when the vacuoles have decreased to minimum in size. These observations were repeated, but yeast-cells which had been stained with congo red were added to the preparations.

The culture from which the paramecia were taken was pH 6.8. They were mounted in this fluid but NaOH was added to some of the preparations, so that a series was obtained which ranged from pH 6.8 to pH 9.8. The stained yeast-cells added were brilliant orange in color, and they remained so in all the preparations. The following results were obtained:

There was no perceptible change in the color in the ingested yeast-cells in any of the preparations until after the food-vacuoles containing them had left the pharynx and had decreased considerably in size. Then, in the preparations, pH 6.8–9, the yeast-cells in vacuoles which contained not more than about six, became sky-blue like buffer pH 3, as the vacuoles decreased in size and then, after the vacuoles had increased in size, brilliant orange again. If the food-vacuoles contained more than about six yeast-cells the extent of change in color from orange toward blue varied inversely with the number of cells in a vacuole. In those which were well filled with yeast-cells and consequently contained but little fluid, there was no appreciable change in color. These vacuoles decreased only slightly in size. The extent of change in color from orange toward blue, i.e., the increase in hydrogen-ion concentration, is therefore correlated with the number of yeast-cells in the vacuoles and the extent of the decrease in their size.

In fluid at pH 9.4 many of the paramecia did not feed, and those which did formed food-vacuoles which were considerably smaller and contained fewer yeast-cells than those formed in ordinary culture fluid. The cells in some of these vacuoles did not appreciably change in color; but those in others became definitely bluish, i.e., about like buffer pH 4. In fluid at pH 9.8 only a few of the paramecia fed and the food-vacuoles formed contained only a few yeast-cells. There was no perceptible change in color in any of these vacuoles, despite the fact that they contained only a few yeast-cells. This seems to show that the extent of change in color from orange toward blue depends upon the alkalinity of the fluid which is ingested with the yeast-cells. The fact, however, that no difference in the extent of change in color was observed in culture fluids which ranged from pH 6.8 to pH 9 indicates that this correlation is not very close.

In some of the observations paramecia were used which had been transferred successively through ten separate portions of fresh sterile culture fluid so as to eliminate nearly all the bacteria. The yeast-cells ingested in this solution changed in color as extensively as those ingested in solutions which contained numerous bacteria. The

increase in the acidity of the fluid in the food-vacuoles is therefore not closely, if at all, correlated with the number of bacteria in it.

There was no perceptible color in the fluid in the food-vacuole in any of the tests made with yeast-cells.

The results obtained with *P. **nucleatum*** and *P. aurelia* are essentially the same as those obtained with *P. caudatum*, but those obtained with *P. trichium* differed in that no change in color whatever was observed in the ingested yeast-cells. In this species the food-vacuoles usually contain only one yeast-cell when they leave the pharynx (never more than two) and very little fluid. They consequently change but little in size after they have left the pharynx. This is probably why there is no perceptible change in the color of the stained yeast-cells in them.

The results obtained with congo red therefore indicate that the maximum acidity of the content of the food-vacuole in *Paramecium* is approximately pH 3.2. This is in harmony with Bozler's conclusion. Nirenstein and Kalmus contend however, as previously stated, that the maximum acidity is much higher.

Nirenstein (1925) found, just as Bozler had, that ingested congo-red-stained yeast-cells become blue in some food-vacuoles in *Paramecium*, but he contends that Bozler's conclusion is erroneous. He put yeast-cells and coagulated egg albumen which had been stained orange with congo red, respectively into different concentrations of hydrochloric acid and found that, whereas a solution of congo red in N/1000 (pH 3) HCl is blue, neither the yeast-cells nor the egg albumen changed color in concentrations lower than N/30 (pH 1.47) and did not actually become blue until N/10 (pH 1) was reached. He consequently concluded that since stained egg-albumen and yeast-cells became blue in some of the food-vacuoles, the acidity of the fluid in those vacuoles must have been approximately pH 1. Kalmus (1931) repeated Nirenstein's observations and came to the same conclusion.

I added yeast-cells and congo red in excess to a series of Clark buffer solutions (pH 1–9) in test-tubes, heated them to boiling and then left them for 12 hours. At pH 2 and lower, the dye had precipitated, the fluid was very light purplish blue and the yeast-cells were not perceptibly stained. At pH 2.2 a few of the yeast-cells were slightly stained, light purple, the same shade as the fluid. At pH 3 the fluid was densely lavender and about one-half of the yeast-cells were strongly stained and the color was like that of the fluid. At pH 5 the fluid and all the yeast-cells were deep red. At pH 7 the fluid was the same color as at pH 5 but the yeast-cells were light orange. At pH 8 and higher the fluid was red but the yeast-cells were only slightly stained and light yellow in color. Congo red therefore appears to stain yeast-cells most readily in the neighborhood of pH 5.

Some of the red yeast-cells in buffer pH 5 were transferred to a series of buffers (pH 1–3) containing congo red and examined from time to time for two hours. At pH 1 and 1.2 the yeast-cells became densely sky-blue, at pH 1.4 purplish blue, at pH 2 and 3 definitely purple.[7]

Some of the red yeast-cells from the pH 5 buffer were added to culture fluid containing numerous paramecia. The yeast-cells were freely ingested. No change in color was observed in any of the food-vacuoles which were well filled with yeast-

[7] It is not clear why yeast-cells do not stain at all with congo red at pH 1 and 1.2 but become densely blue at these hydrogen-ion concentrations if they are first stained at higher concentrations.

cells, but in some of those which contained only a few, they unquestionably became as blue as those in buffers pH 1 and 1.2 and then, as the vacuoles increased in size, red again. In others they became purple of various shades so that all colors obtained in buffers pH 1–3 could be matched.

These results appear therefore to support Nirenstein's conclusion that the maximum acidity of the content of some of the food-vacuoles in *Paramecium* is approximately pH 1. The salt, protein and other errors for congo red are however so great that Clark (1928) and others maintain that the results obtained with it can be considered only as crude approximations. Moreover, in measurements made with a glass electrode, I found that congo red decreases the acidity of solutions and that in saturated solutions of congo red this decrease is great, i.e., from pH 3.06 to pH 6.61 in N/1000 HCl. Nirenstein's conclusion regarding the maximum acidity of the content of the food-vacuole, based on the assumption that congo red does not affect the acidity of solutions of hydrochloric acid, is consequently equivocal. I therefore repeated the experiments on congo red described above, with thymol blue and meta cresol purple, both of which have only very small errors and only slightly affect the acidity of solution to which they are added.

Thymol blue (pH 1.2, red—pH 2.7, yellow) and *meta cresol purple (pH 1.2, red—pH 2.8, yellow)*

In buffer solutions containing thymol blue, yeast-cells stain most readily at pH 3–5 and become lemon yellow. At pH 1–1.6 they do not stain perceptbily, but if yellow yeast-cells are put into these buffer solutions they become definitely pink of the same shade as the solutions.

Paramecia ingest the stained yeast-cells freely except in preparations in which the dye is too concentrated or the fluid too strongly acid. In every preparation studied the density of the yellow color of the yeast-cells in the forming food-vacuoles increased considerably. This is doubtless due to increase in the concentration of the dye, owing to impermeability of the vacuolar membrane to it and loss of water. After the food-vacuoles had left the pharynx, the yeast-cells in most of the vacuoles remained yellow but in some in nearly every preparation they unquestionably changed color and became as pink as those in buffer pH 1.4, and then as the vacuoles increased in size, yellow again.

The results obtained with meta cresol purple are essentially the same as those obtained with thymol blue. The ingested, stained yeast-cells remained yellow in most of the food-vacuoles, but in some they changed color as the vacuoles decreased in size and clearly became as pink as those in the buffer pH 1.4, and then yellow again as the vacuoles enlarged.

The results presented seem to demonstrate that while the maximum acidity of the content of the food-vacuoles in *Paramecium* differs enormously, it is at least as high as pH 1.4 in some of them. If this is true it is phenomenal, for culture fluid very much lower (even as low as pH 3.5) is instantly fatal to paramecia. The relatively high concentration of acid in the food-vacuole without injury to the adjoining cytoplasm is convincing evidence in support of the contention presented above that the vacuolar membrane is impermeable to acid.

The Maximum Alkalinity of the Content of the Food-Vacuole

Four dyes were used in the observations on the maximum alkalinity of the content of the food-vacuoles in *Paramecium*, nile blue (Grübler, 1915, pH 7, blue—pH 8, purple); phenol red (pH 6.8, yellow—pH 7.4, pink); brom thymol blue (pH 6, yellow—pH 7.6, blue), and cresol red (pH 7.2, yellow—pH 8.8, red). The observations made with congo red were repeated with each of these four dyes except that only two species (*P. caudatum* and *P. nucleatum*) were used and that the hydrogen-ion concentration of the culture fluid in which the paramecia were mounted, extended over a wider range. No difference was observed in the results obtained with the two species.

Nile blue

In the paramecia mounted in culture fluid containing nile blue, numerous food-vacuoles with many bacteria were formed in all the preparations except those which were so strongly alkaline that they were definitely toxic. These preparations were examined under low and high magnification from time to time for six hours. The content of the food-vacuoles was practically colorless until after the vacuoles had decreased considerably in size; then the bacteria in them became deep sky-blue in all the preparations and appeared to be dead, but the fluid remained colorless. Later the vacuoles increased in size, but there was no appreciable change in the color of either the fluid or the bacteria in them. There was no indication of a change to a purplish tint in the content of any of them. The buffer solutions containing Grübler's nile blue were sky-blue at pH 6.8, slightly purplish at pH 7.2, and distinctly purple at pH 7.6 and higher. The results presented, therefore, seem to show that the content of the food-vacuoles certainly did not become as alkaline as pH 7.6 in any of them, and probably not as alkaline as pH 7.2.

Yeast-cells stained with nile blue were mounted respectively in acid (pH 6.8) and alkaline (pH 8.6) culture fluids containing paramecia. The yeast-cells were freely ingested by the paramecia in all the preparations studied. In the acid culture fluid they were sky-blue and no perceptible change in color occurred as they passed through the body. In the alkaline culture fluid they were purple and they usually remained purple, but occasionally, if the food-vacuoles contained only one or two, they became blue as the vacuoles decreased in size and then purple again after they had enlarged.

Some of the blue yeast-cells were mounted in each of the buffer solutions in the series prepared. They remained blue in all below pH 7.6 and became only slightly purplish at pH 7.8. The results presented above indicate, therefore, that the content of some of the food-vacuoles formed in alkaline culture fluid became at least as alkaline as pH 7.8; but the fact that the yeast-cells became purple in only a very small percentage of the food-vacuoles shows that it rarely becomes as alkaline as this. However, the fact that the yeast-cells did not become purple in any of the vacuoles formed in acid culture fluid indicates that the maximum alkalinity of the content of the food-vacuoles is correlated with the hydrogen-ion concentration of the surrounding medium.

Phenol red

Paramecia can withstand a surprisingly strong solution of phenol red. If the solution is alkaline the cytoplasm becomes distinctly yellowish green. The bacteria in the culture fluid used containing phenol red were colorless and the fluid was yellow or pink, depending upon the hydrogen-ion concentration. In the food-vacuoles the bacteria, regardless of the hydrogen-ion concentration of the culture fluid, became dark yellow as the vacuoles decreased in size, but the fluid around them remained colorless. Then as the vacuoles enlarged the fluid and the bacteria became faintly but distinctly pink (pH 7.4) in some of them and colorless in the rest. These results are essentially in accord with those obtained by Shipley and DeGaris (1925).

Stained yeast-cells became distinctly yellow in the acid and distinctly pink in the alkaline culture fluids used. In the food-vacuoles the pink yeast-cells became yellow and the yellow ones remained yellow as the vacuoles decreased in size, the fluid remained colorless. In about five percent of the vacuoles, especially those which contained only a few yeast-cells, both kinds became faintly but distinctly pink in color similar to stained yeast-cells in buffer pH 7.4, as the vacuoles enlarged. In the rest they remained either yellow or became colorless. No definite correlation between the hydrogen-ion concentration of the surrounding medium and the changes in the color of the content of the vacuoles was observed.

These results indicate that the maximum alkalinity reached in the food-vacuoles varies greatly in different vacuoles but that it never exceeds pH 7.4. Phenol red is, however, not very satisfactory for the measurement of the hydrogen-ion concentration of the content of food-vacuoles; for the changes in color are rather indefinite and the color of buffer solutions is at best not closely correlated with their hydrogen-ion concentration.

Brom thymol blue

The bacteria in the culture fluid, both acid and alkaline, containing brom thymol blue were colorless. They were consequently colorless when they entered the food-vacuoles. No changes in color in them or in the fluid around them was observed as they passed through the body.

In the alkaline culture fluid used the stained yeast-cells became blue, and in the acid culture fluid yellow. The blue yeast-cells which were ingested became yellow almost immediately after the food-vacuoles left the pharynx, and in all the food-vacuoles which contained more than about four, they remained yellow until they were eliminated or became colorless; but in the food-vacuoles which contained fewer than about four they became (after the vacuoles had enlarged) greenish blue, about the shade assumed by stained yeast-cells in buffer pH 7 or 7.2. No difference in shade was observed in the acid and the alkaline culture fluids used.

These results indicate, therefore, that the maximum alkalinity attained by the content of the food-vacuoles is not greater than pH 7 or possibly 7.2, i.e., not so great as the results obtained with nile blue and phenol red indicate. The changes in the color of yeast-cells stained with brom thymol blue is, however, so elusive in the food-vacuoles that they indicate but little concerning the hydrogen-ion concentration of the fluid around them.

Cresol red

The bacteria in culture fluid containing cresol red were colorless when they were ingested, and no change in color was observed in them as they passed through the body.

The stained yeast-cells became light yellow in the acid culture fluid used and light pink in the alkaline. In the food-vacuoles they remained or became yellow as the vacuoles decreased in size and then, with a very few exceptions, either became colorless or remained yellow until they were eliminated, i.e., they did not change color after the vacuole had enlarged. In a few instances, however, the yellow yeast-cells assumed a light pink color, about like buffer pH 7.8.

It is evident that the results obtained with the four dyes used differ considerably. They seem to indicate however that the alkalinity of the content of some of the food-vacuoles is nearly, if not quite so high as pH 7.8 but that it is much lower in most of the vacuoles. They also indicate the maximum alkalinity varies somewhat with the acidity of the fluid ingested.

The Origin of the Acid and the Base in the Food-Vacuole

It is widely held that the acid and the base in the food-vacuole are secreted by the adjoining cytoplasm (Greenwood and Saunders, 1894; Nirenstein, 1905; Lund, 1914; Howland, 1928; Claff et al., 1941; and others). However, Mast (1942) concludes that in *Amoeba* the acid originates in the vacuoles and increases in concentration owing to impermeability of the vacuolar membrane to the acid and loss of water, and that the subsequent increase in alkalinity is due to diffusion of alkaline fluid from the cytoplasm into the vacuoles, that is, that "the cytoplasm secretes neither acid nor base." And Mast and Bowen (1944) conclude that in the Peritricha, "The increase in the acidity of the content of the vacuole is probably due to the production of acid, owing to metabolism in the peristome, the vestibulum and the pharynx and impermeability of the vacuolar membrane to organic acid, resulting in its retention and consequent concentration as the vacuole decreases in size." Mast and Bowen (p. 213) present strong evidence in support of their conclusion. This evidence applies equally well to the food-vacuoles in *Paramecium* and leads to the same conclusion. It should be emphasized, however, that secretion of acid by the cytoplasm adjoining *the vestibulum and the pharynx* is by no means ruled out.

The increase in alkalinity in the food-vacuole in *Paramecium* is doubtless brought about in the same way as it is in the Peritricha, i.e., by entrance of alkaline fluid from the cytoplasm. According to these views the cytoplasm adjoining the food-vacuoles does not secrete either acid or base.

The Function of the Changes in the Hydrogen-ion Concentration in the Food-Vacuole

It is maintained by Hemmeter (1896), Nirenstein (1925), Howland (1928), and Claff et al. (1941) that the organisms ingested by protozoa are killed by the acid in the food-vacuoles and that this is its primary if not its only function. There is, however, strong opposition to these contentions.

Fortner (1933) holds that in *Paramecium caudatum* the ingested organisms are killed by a toxic substance. He asserts that this substance is formed in the neutral-

red granules, and that these granules pass through the vacuolar membrane into the food-vacuole shortly after it has left the pharynx, and release the substance there.

These assertions are certainly not strictly valid, for as demonstrated above, the neutral-red granules do not enter the food-vacuole. It might be, however, that since some of them are in close contact with the vacuolar membrane when the food-vacuole leaves the pharynx, toxic substance diffuses from them into the vacuole and kills the ingested organisms. It is however far more likely that if toxic substance is involved in this phenomenon, it is secreted by the wall of the pharynx and taken up by the fluid which passes into the forming food-vacuoles, and that it there (owing to impermeability of the vacuolar membrane and loss of water) increases in concentration and consequently becomes lethal. At any rate, there is much evidence that the pharynx secretes substances which pass into the forming food-vacuole.

Fortner (1933) found that if numerous crushed bacteria are added to culture fluid containing live bacteria and paramecia the ingested bacteria are not killed, and that the vacuoles, as previously stated, do not decrease in size. This seems to show that death of the bacteria depends upon decrease in the size of the vacuoles. If this is true, it lends support to the hypothesis that the bacteria in the food-vacuole are killed by toxic substance which has been concentrated in the food-vacuoles owing to loss of water and impermeability of the vacuolar membrane to the toxic substance.

Mast (1942) in observations on *Amoeba* and Mast and Bowen (1944) in observations on the Peritricha present strong evidence that the acid in the food-vacuoles in these protozoa does not become sufficiently concentrated to kill the ingested organisms and the results presented above seem to show that this also obtains for most of the food-vacuoles in *Paramecium*. Mast concludes that in *Amoeba* the primary lethal factor in the food-vacuole is reduction of oxygen, owing to respiration and loss of water, and Mast and Bowen accept this view in reference to the Peritricha, but they present no evidence in support of it and none was obtained in the present observations on *Paramecium*.

No one has observed any indication of digestion in the food-vacuole during its acid phase. Nirenstein (1905) thinks, however, that there are invisible preliminary changes. However this may be, it is practically certain that the osmotic concentration of the fluid in the vacuole increases greatly during this phase, and it may well be that this is caused by hydrolysis of complex compounds, owing to the presence of acid, and that this causes the rapid inflow of fluid from the cytoplasm, which probably contains digestive enzymes. If this is true, the acid in the food-vacuoles obviously functions, at least indirectly, in digestion. There are consequently several hypotheses concerning the function of the acid in the food-vacuole but there is not much evidence in support of any of them.

It has been conclusively demonstrated that digestion in the protozoa takes place largely, if not entirely, in the food-vacuoles during the alkaline phase, but since this phase seems to be merely the result of inflow of fluid from the cytoplasm, digestion is not correlated with anything in the nature of secretion of alkaline substance.

Digestion

Nirenstein (1905) made detailed observations on bacteria, yolk granules, fat globules, and starch grains in the food-vacuoles in *Paramecium caudatum*. He says he observed no indication of digestion in any of these substances until after the fluid

in the vacuoles had become alkaline, but that after this had occurred the bacteria and the yolk granules gradually decreased in number and finally disappeared entirely and some of the starch grains corroded. He was at this time in doubt about the fate of the fat globules, but he later (1910) presented evidence which strongly indicates that fat is hydrolyzed in the food-vacuoles.

Nirenstein's views concerning the time of digestion in the food-vacuoles in *Paramecium* and the digestion of proteins have been adequately confirmed; his contention that paramecia digest starch is supported by results obtained by Pringsheim (1928) and Zingher (1933) in observation on *Paramecium caudatum;* and his contention that they digest fat is indirectly supported by results obtained by Dawson and Belkin (1929), Mast (1938), and Wilber (1942) in observations on *Amoeba* and *Pelomyxa.*

I repeated Nirenstein's observations and obtained results which are in harmony with his. It should be emphasized, however, that in my experiments only a very small proportion of the starch (wheat and potato) ingested by the paramecia was digested. Observations by Greenwood (1886), Meissner (1888), and Mast and Hahnert (1935) indicate that this also obtains for *Amoeba.* Indeed, it is doubtful whether in this organism any starch is digested except that which is in other organisms which have been ingested.

The Origin of the Digestive Enzymes and the Function of the Neutral-Red Granules and the Mitochondria

The fact that protein, starch and fat are digested in the food-vacuoles in *Paramecium* shows that these vacuoles contain peptidase, amylase, and lipase, and the fact that digestion does not begin until after the fluid in the vacuole has become alkaline shows that these enzymes are active in a basic medium. The questions now arise as to where these enzymes originate and how they get into the food-vacuoles.

Prowazek (1898) who, in observations on paramecia, discovered the neutral-red granules and their tendency to aggregate at the surface of the forming food-vacuole, maintains that they contain digestive enzymes. He consequently called them "Fermentträger". He offers no explanation as to how the postulated enzymes in the granules get into the vacuole, but Nirenstein (1905) who accepts Prowazek's contentions, holds that the granules pass through the vacuolar membrane and carry the enzymes into the vacuole. He asserts that, in paramecia in culture fluid containing neutral red, he observed red granules appear in food-vacuoles shortly after they had left the pharynx. He consequently concluded that the red granules in the food-vacuoles came from the cytoplasm and passed through the vacuolar membrane into the food-vacuoles and that the enzymes in the vacuoles are carried in by these granules.

These conclusions are supported by Rees (1922), Bozler (1924), Fortner (1926), Volkonsky (1929), Müller (1932), and MacLennan (1941, p. 129). All these investigators hold that in *Paramecium* the neutral-red granules actually pass through the vacuolar membrane and carry enzymes into the food-vacuoles. None of them, however, maintains that he saw this. Their contention is based largely on Nirenstein's observation of the appearance of red granules in the food-vacuoles. It is, however, obvious that these granules may have entered the food-vacuoles by way of the pharynx. The evidence in support of the contention that neutral-red

granules pass from the cytoplasm into the food-vacuoles in *Paramecium* is therefore very weak. There is, however, evidence which strongly indicates that in some other organisms neutral-red staining substance actually passes from the cytoplasm into the food-vacuoles.

MacLennan (1936) asserts that in *Ichthyophthirius* neutral-red staining substance becomes attached to particles of food which are naked in the cytoplasm, that these particles aggregate, and that a membrane then forms, enclosing them, together with some fluid, in a vacuole. He thinks that the neutral-red staining substance contains digestive enzymes. Hopkins and Warner (1946) hold similar views regarding the food-vacuoles in *Entamoeba histolytica* and they maintain, moreover, that they actually saw globules pass through the vacuolar membrane into and out of the food-vacuoles.

The idea that granules or globules carry enzymes into the food-vacuoles, is consequently widely held. There is, however, evidence which very strongly indicates that this does not obtain in *Paramecium.*

Khainsky (1911) studied the food-vacuole of *Paramecium caudatum* in fixed and stained specimens and in living specimens. He obtained no evidence which indicates that granules or globules pass from the cytoplasm into the food-vacuoles. He maintains, however, that neutral-red staining globules form in the vacuole and pass out through the vacuolar membrane. Koehring (1930) made extensive observations on neutral-red granules in various protozoa. She concludes that they function as enzyme carriers in all, but that they do not enter the food-vacuoles. Referring to *Paramecium caudatum* she says (p. 67): "As the new vacuole is being formed they [the neutral-red granules] gather at the membrane, bombarding it like hailstones, but making no impression on the firm surface. Then as this vacuole flows away attached by its canal . . . some of the granules leave this vacuole and return to the next, which is already in the process of formation. Those left continue bombardment of the vacuole as the pink color slowly forms within."

Dunihue (1931) asserts that in *Paramecium caudatum* the surface of the forming food-vacuoles becomes "closely packed" with neutral-red staining globules "which remain firmly attached throughout most of the digestive period" and do not enter the vacuoles.

As stated in a preceding section, I made under most favorable conditions intensive observations on the neutral-red granules at the surface of food-vacuoles throughout their entire existence and obtained no evidence indicating that visible granules or globules of any kind ever pass through the vacuolar membrane into or out of the food-vacuoles in *Paramecium.* What, then, was the origin of the red granules observed by Nirenstein in the food-vacuoles in *Paramecium?*

These granules doubtless originated in substance ingested by the paramecia. At any rate it has been clearly demonstrated that this occurs in *Amoeba proteus* (Mast and Hahnert, 1935; Mast and Doyle, 1935). It can therefore be concluded that the neutral-red granules found in the cytoplasm in *Paramecium* do not enter the food-vacuoles. This does not, however, demonstrate that they are not involved in digestion. If they contain enzymes it may be that in aggregating at the surface of the food-vacuoles the concentration of enzymes is increased and that this facilitates their diffusion into the vacuoles. The facts, however, that there is a marked flow of fluid out of the vacuole during the entire time that the aggregation of these granules is prominent and that most of the granules usually leave the surface of the vacuoles

long before digestion begins, seem to militate against this possibility. Moreover, Hall and Dunihue (1931) and Mast and Bowen (1944) found that in *Vorticella* and other Peritricha the neutral-red granules do not aggregate at the surface of the food-vacuoles, and Mast (1926) found that in *Amoeba* there are none. They are therefore certainly not functional in digestion in the latter and very probably not in the former. The evidence in support of the contention that they function in digestion in *Paramecium* is therefore negligible. What, then, is their function in this organism?

Fortner (1926, 1928) maintains that they originate in the cytoplasm adjoining the nucleus, that they contain protein and fat, and that they decrease greatly in number during starvation. If these contentions are valid, the composition of the granules and their disappearance seem to suggest that they are reserve food; but, if this is what they are, why do they originate near the nucleus, and why do they aggregate at the surface of the food-vacuole during its formation? The function of the neutral-red granules in *Paramecium* is apparently still decidedly hazy.

Horning (1926a) contends that in *Amoeba sp.* janus-green staining bodies (mitochondria) aggregate among particles of food in the cytoplasm and that a membrane then forms and encloses the food and the mitochondria in a vacuole. He says that in *Paramecium sp.* mitochondria "are extruded from the cell protoplasm [through the vacuolar membrane] into the food vacuoles" during the alkaline phase. He concludes that the mitochondria in these protozoa function in carrying digestive enzymes to the ingested food. Volkonsky (1934) does not agree with Horning. He maintains that the mitochondria do not enter the food-vacuoles and that they do not take part in digestion in protozoa.

As stated above, I was unable to see any particles pass from the cytoplasm into the food-vacuoles in *Paramecium*. Hall and Nigrelli (1930) could not obtain any evidence of mitochondria in the food-vacuoles in *Vorticella*, and Mast and Doyle (1935) in an intensive study of the movement of the mitochondria in *Amoeba proteus* under very favorable conditions concluded that they do not pass into the food-vacuoles. They found that the mitochondria move about in the cytoplasm freely and often come in contact with the surface of the food-vacuoles in all stages of development and at times form aggregations at the surface, but that none pass into the vacuoles. These observations and others led them to conclude that the mitochondria in *Amoeba* "probably function in transferring substances from place to place in the cytoplasm."

Holter and Kopac (1937) maintain that in centrifuged specimens of *Amoeba proteus*, the mitochondria are in a fairly well defined stratum but that the enzyme, peptidase, is not stratified. They consequently conclude that this enzyme is not associated with mitochondria. However, Holter and Doyle (1938) in similar tests found that amylase and mitochondria in *Amoeba* tend to accumulate in the same stratum. These results seem to indicate that this enzyme is attached to the mitochondria. The authors do not, however, hold that this association is proved.

The evidence in support of the contention that the mitochondria and the neutral-red granules in *Paramecium* are enzyme carriers is therefore, as yet, far from convincing. Where, then, do the digestive enzymes in the food-vacuoles come from?

Some doubtless come from the organisms and other substances which have been ingested and the rest are, I think, as previously stated, carried from the cytoplasm into the vacuoles with the fluid that enters during the rapid enlargement of the vacuoles at the beginning of the alkaline phase. Where and how the enzymes in the

cytoplasm originate is not known. Fortner (1926), as stated above, maintains that the neutral-red granules are formed in the cytoplasm adjoining the nucleus. He holds that the enzymes are closely associated with these granules and consequently concludes that they also originate in this region. The validity of the contention that the enzymes are closely associated with the neutral-red granules is, however, very doubtful. The evidence in support of Fortner's conclusion is, therefore, very weak. It is known however that in *Chilomonas paramecium* enzymes are synthesized in the protoplasm, for this organism can be grown on known simple chemical compounds (Mast and Pace, 1933), but how and where they are synthesized and what they are, chemically, is not known, and this can also be said of the enzymes in other protozoa.

Summary

1. The feeding apparatus in *Paramecium* consists of a shallow ciliated groove, a ciliated tube which leads into the body, and a bundle of fibers (esophageal fibers) which extend from the tube nearly to the posterior end of the body. The tube is composed of an outer part (the vestibulum) and an inner part (the pharynx).

2. Paramecia ingest all sorts of small particles, but more digestible than indigestible ones. Selection takes place in the vestibulum and the proximal end of the pharynx.

3. In forming a food-vacuole, the cilia in the pharynx force fluid with particles in suspension against the membrane over the distal opening of the pharynx, producing a sac, the esophageal sac.

4. As the esophageal sac enlarges, the particles in suspension in it become greatly concentrated, owing largely, if not entirely, to the passage of water out through the membrane into the cytoplasm.

5. A portion of this sac is constricted off, as a food-vacuole, probably by the action of the esophageal fibers.

6. The initiation of the constriction of the sac is probably due to periodicity in the constrictive action of the fibers, the size of the sac, and the composition of its contents.

7. There is much variability in the size of the food-vacuoles. This is correlated with the quantity and the quality of the particles in the surrounding fluid, the chemical composition of this fluid, the rate of ingestion, the rate of loss of water from the esophageal sac, and the length of the intervals between consecutive constrictions of the esophageal fibers. The frequency of formation of food-vacuoles is correlated with the quantity and the quality of the particles in the surrounding fluid and the acidity, and the temperature of this fluid. The shape of the food-vacuoles depends largely, if not entirely, upon the viscosity of their content.

8. After the food-vacuole has left the pharynx it passes rapidly on a fixed course toward the posterior end of the body, and slowly on a varied course to the anus. The former is probably due to the action of the esophageal fibers; the latter is due to cyclosis.

9. On its course through the body, the food-vacuole usually decreases greatly in size, and the acidity of its content increases greatly; then it enlarges very rapidly and the acidity of its content decreases greatly. The extent of these changes varies enormously. Under some conditions there are no perceptible changes; under others

the acidity in some vacuoles increases to a maximum at least as high as pH 1.4 and then decreases approximately to pH 7.8.

10. There is no "preliminary alkaline phase" of the food-vacuole.

11. The change in acidity is definitely correlated with change in size. The changes in size are due to difference between internal and external osmotic concentration and the action of the stretched vacuolar membrane. The increase in acidity is probably due to secretion of acid by the cytoplasm adjoining the vestibulum and the pharynx and to impermeability of the vacuolar membrane to hydrogen-ions, and loss of water. The decrease in acidity is due to entrance of alkaline fluid from the cytoplasm.

12. The increase in acidity probably causes hydrolysis and thereby increase in osmotic concentration resulting in inflow of fluid containing digestive enzymes.

13. Death of ingested living organisms is probably largely due to toxic substance produced by the pharynx and concentrated in the food-vacuole, owing to impermeability of the vacuolar membrane to it, and loss of water.

14. Paramecia digest protein, fat, and starch. Digestion takes place during the alkaline phase of the food-vacuole. The enzymes involved originate in the cytoplasm and are carried into the food-vacuole by the cytoplasmic fluid which enters during its rapid enlargement.

15. The neutral-red granules and the mitochondria are probably not involved in digestion.

16. All these phenomena are essentially the same in the four species studied, namely *P. caudatum, P. nucleatum, P. aurelia,* and *P. trichium.*

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POISONING AND RECOVERY IN BARNACLES AND MUSSELS[1]

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Introduction

Previous investigations have shown that the prevention of the attachment of organisms to ships' hulls by antifouling paint is related to the rate of solution of the toxic material, and hence, to its concentration in the sea water at the paint's surface (Ketchum, Ferry, Redfield, Burns, 1945). The present investigation was undertaken to test the effectiveness of different metallic salts as poisons for two important types of fouling organisms and to evaluate them under conditions in which the concentration and ionic form of the toxic materials were accurately known. In earlier attempts to test lethal action, supposedly toxic materials have been incorporated in paints applied to test-panels or to the inside surfaces of containers and the degree of fouling resistance noted, but in most cases no adequate measure was made of the concentrations of the toxics which were actually emitted by the paint. In other tests, fouling organisms were placed in containers of sea water to which known amounts of toxics had been added but without adequate knowledge of the ionic transformation of the toxics which ensue in the presence of sea water (Bray, 1919; Edmondson and Ingram, 1939; Orton, 1929–30; and Parker, 1924).

We proposed, therefore, to conduct direct tests of the action of various individual metals in graded concentrations and in different chemical forms, on two important types of fouling organisms; namely, barnacles and mussels. We desired also to investigate the relationship between the concentration of various toxics and the exposure time necessary to kill, in order to ascertain the most efficient concentration of the poison for the destruction of fouling organisms.

A second purpose was to follow the course of poisoning and recovery in barnacles and mussels when copper was used as the poison. It was desired to measure the amount of copper in the tissues and to discover whether death from copper poisoning was brought about specifically by the accumulation of a certain amount of the poison in the tissues or by the entrance of the copper into the body at a certain rate or for a critical period of time. Finally, it was desired to ascertain from how great a dose of copper an animal could recover, and to learn whether, after recovery, the animal was more or less susceptible to subsequent exposures of the toxic.

I am indebted to Mrs. Dayton Carritt for assistance in carrying out the experiments, to Mrs. Barbara Mott Ferry for chemical analyses, to Dr. John Ferry and Dr. Gordon Riley for furnishing toxics, and to Mr. Porter Smith for technical assistance. I am indebted to the University of Miami for the use of facilities at the

[1] This investigation was made for the Woods Hole Oceanographic Institution under contract with the Bureau of Ships, Navy Department, which has given permission for its publication. The opinions presented here are those of the author and do not necessarily reflect the official opinion of the Navy Department or the naval service at large.

[2] Contribution Number 360.

Marine Laboratory. I also wish to thank Dr. Walton Smith and Dr. Charles Pomerat for valuable cooperation.

Observations on Adult Barnacles and Mussels

The effectiveness of various poisons was investigated first using the barnacle as a test animal, since the barnacle is generally the most serious offender as a fouling organism. Adult barnacles were employed for the majority of the experiments at Woods Hole, but one set of tests was conducted on nauplii which had been discharged in a laboratory tank. Although attachment of the barnacle takes place previous to the adult stage, there is evidence that a poisoned barnacle is less securely attached than a normal animal and may be more easily dislodged. Special interest centers about the poisoning of the cyprid, since this is the attaching stage. In view of the fact that cyprids could not be obtained in sufficient numbers at Woods Hole, experiments on this stage were conducted at Miami Beach, Florida. Mussels, which are of relatively large size, were found advantageous for other tests, especially those involving the copper assay of living tissue.

Source and preparation of material

Certain of the present tests were carried out with *Balanus balanoides* growing on stones obtained near low tide mark at Woods Hole. In later tests *Balanus eburneus* was employed, obtained through the kindness of Dr. Stanley Cobb, from a wharf in Cotuit Harbor. Parts of the wharf were removed and transported to the laboratory tanks. The barnacles were kindly identified by Dr. Richard McLean.

For the experiments with *B. balanoides* the material was collected on the same day that the tests were begun. Single stones bearing 10 to 30 barnacles (ranging from 3 mm. to 10 mm. in diameter) were placed in individual beakers or culture dishes containing from 250 cc. to 500 cc. of sea water with or without toxic. For the work using *B. eburneus,* a stock of animals attached to fragments of wood was maintained in large battery jars provided with running sea water. In preparation for the tests it was necessary to split and saw the pieces of wood into blocks of convenient dimensions. Blocks about 4 cm. to 6 cm. square, each bearing 10 to 20 barnacles ranging from 4 mm. to 20 mm. in diameter, were similarly placed in individual containers. In other experiments larger blocks bearing several hundred barnacles were placed in battery jars of 8 liters capacity or more.

With both species of barnacles it was found that when the sea water was renewed daily, the animals in the individual containers remained in a healthy condition for several weeks. There were evidently enough food particles in the sea water supplied to provide a maintenance diet. Faecal material was produced in abundance but the barnacles grew little, if at all.

The mussels employed were the common species in the Woods Hole region, *Mytilus edulis,* obtained from the dock and float of the Oceanographic Institution. The mussels were placed individually or in batches in culture dishes or large battery jars. The animals began attaching their byssus threads to the bottoms or sides of the containers almost immediately. If the sea water was renewed each day, the mussels continued in healthy condition indefinitely, apparently obtaining sufficient nutriment from the food particles present in the sea water.

Behavior during poisoning

When barnacles were placed in the toxic media, they opened their shells and began raking or filtering the water with their cirri as soon as did the controls in fresh sea water. Ordinarily this took place within a few minutes. If the concentration of the poison was such as to kill the barnacle within two or three days, the animals would begin to show signs of bad condition after about 12 hours. The first symptom was the slowing down of the beat of the cirri and soon thereafter their sweep became reduced in extent. As poisoning progressed, the movement of the cirri became still slower and more curtailed until they opened to only one-half or one-quarter of their usual scope. When the poisoned barnacle stopped filtering, it tended to come to rest with the cirri half extended, in contrast to the healthy animal in which the cirri are drawn completely in and the shell tightly closed during rest. In the poisoned animal, the shell could at first be caused to close by prodding it with a needle. After further poisoning, when no response could be elicited by stimulation with the needle, the barnacle was considered dead. The following symbols are used in the tables to designate these conditions:

++++	normal rapid sweep of cirri
+++	full sweep but slow
++	incomplete sweep
+	open, inactive, but reacts to touch
0	dead

If the barnacle was replaced in fresh sea water (preferably running sea water) within a day of the time it reached the next to last condition (+), it would usually recover completely its full activity after a few days.

In the case of the mussel, the change in condition as poisoning progressed was less easily observed. In the higher concentrations of the toxics, the animals failed to attach themselves to the walls of the containers and tended to remain closed for long periods. When placed in weaker solutions, however, many of the mussels became strongly attached and remained with their shells open to the normal extent to allow the inflow and outflow of water. In either case, after poisoning had progressed to a certain point, the valves were found gaping wide open. When this condition was first reached, the animal responded to prodding by closing slowly. A day later, or less, no response would follow such stimulation and the animal was considered dead. Once the mussel reached the stage in which it gaped open more than normal, though it might still react, it would not recover when placed in fresh sea water. In fact, mussels were frequently observed to die many days after having been transferred from the toxic solution into fresh sea water although outwardly they appeared to be in normal condition in the interim.

Toxicity of various metallic salts to barnacles

The toxicity of the following metals was investigated: copper, mercury, silver, and zinc. The last three of these was tested as the ions which resulted from the introduction into the sea water of $HgCl_2$, $AgSO_4$, and $Zn(NO_3)_2$ respectively. The copper, on the other hand, was tested not only in the form of basic cupric carbonate, but also as cupric citrate, cupric tartrate, cupric salicylate, and cupric para amino-

benzoate. The interest in these other compounds of copper lies partly in the possibility of revealing important differences in toxicity. Of further significance is the fact that these substances are much more soluble in sea water than is basic cupric carbonate. Therefore, when copper is supplied in the form of these more soluble ions, it is possible to establish a high concentration of the toxic in the water adjacent to the surface from which the material is free to dissolve.

Individuals of *Balanus balanoides* were placed in beakers or jars containing graded concentrations of toxics in sea water with a control for each series. Since the media were renewed each day, the danger of significant changes in concentration due to such causes as adsorption was minimized. The minimum concentrations necessary to kill 90 per cent of the barnacles after continuous exposures of two days and five days are indicated in Table I. Among the various forms of copper, the

TABLE I

The concentrations of various metallic salts necessary to kill 90 per cent of adult barnacles after continuous exposures for the indicated periods. Concentrations are expressed as milligrams of metal per liter of sea water

	Toxic	Concentration lethal in 2 days	Concentration lethal in 5 days
		mg./liter	mg./liter
Series I			
	Basic Cu Carbonate	0.35	—
	Basic Cu Carbonate	0.48	—
Balanus balanoides	Cu Citrate	0.60	0.18
from	Cu Citrate	0.60	0.30
Woods Hole harbor	Cu Tartrate	0.58	0.17
	$HgCl_2$	1.0	0.5
	Ag_2SO_4	0.4	0.2
	$Zn(NO_3)_2$	32.	8.0
Series II			
	Basic Cu Carbonate	0.28	0.14
Balanus eburneus	Cu citrate	0.55	0.14
from	Cu salicylate	0.90	0.45
Cotuit harbor	Cu para aminobenzoate	—	0.50

basic cupric carbonate was the most effective but the concentrations of the citrate and tartrate necessary to kill were only slightly greater. The toxicity of silver as silver sulphate was of the same order of magnitude but somewhat greater concentrations of mercury as mercuric chloride were required. The effectiveness of zinc, on the other hand, was very much less than any of the other substances tested (*cf.*, Riley, 1943).

Experiments with *Balanus eburneus* indicated similarly that the lethal action of cupric citrate is somewhat less than that of basic cupric carbonate although the magnitude of the difference is not great. The toxicity of cupric salicylate and cupric aminobenzoate were somewhat less than the citrate.

In view of the fact that the toxicity of cupric citrate was found to be almost as great as that of basic cupric carbonate, and since stock solutions of higher and less

variable concentrations could be maintained, the tests which follow were conducted with the citrate.

Relation between concentration of cupric citrate and killing time

Barnacles

With the higher concentrations of cupric citrate in which killing occurred within a few days, adult barnacles all succumbed at about the same time, but at the lower concentration it was difficult to determine accurately when the barnacles should be considered dead. At still greater dilutions of the toxic, it was found that most of the animals lived on in a feeble condition for two or three weeks or more. Furthermore, there was considerable variation from experiment to experiment, which was no doubt due not only to individual differences in the batches of barnacles, but also

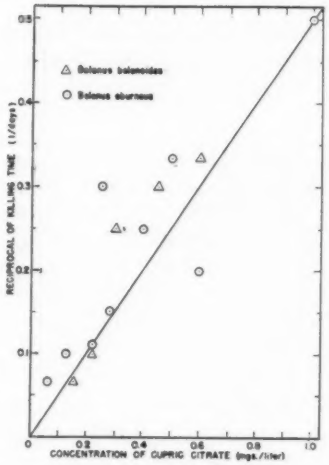

FIGURE 1. The relation between killing time and concentration of toxic for two species of barnacles.

to unavoidable changes in the seasonal condition and in the age of the animals. No consistent trend appeared in relation to the latter conditions except that barnacles in the nauplius stage were killed much more quickly than the adults, as noted below.

For both *B. balanoides* and *B. eburneus*, the killing time ranged between two and five days for concentrations of 0.60 to 1.00 mg. Cu/liter. At 0.22 mg./liter the animals succumbed after ten days. At concentrations of 0.06 mg./liter and less, the animals remained alive for two weeks or more. When the reciprocal of the killing time was plotted against the concentration, as in Figure 1, a roughly linear relation was found. The approximate linearity of this relation indicates that the rate of action of the poison is directly proportional to its concentration.

The results of a series of tests on the poisoning of nauplii, made on the day of their release from the brood pouch, are shown in Table II. They indicate that the

TABLE II

Effect of concentration of cupric citrate on the survival of nauplii of Balanus eburneus. August 25–27, 1942

Concentration mg. Cu/liter	After 22 hrs.	After 29 hrs.	After 48 hrs.
1.00	all dead	—	—
0.50	all dead	—	—
0.25	all dead	—	—
0.13	75% alive	all dead	—
0.06	75% alive	50% alive	1% alive
0.03	100% alive	95% alive	5% alive
Control	100% alive	95% alive	5% alive
Control	100% alive	95% alive	10% alive

nauplii are killed after much shorter exposure than is the case with the adults. The test was not very satisfactory because the controls lived for only about two days.

Mussels

The experiments on the mussel, *Mytilus edulis,* indicate that it is much more sensitive to cupric citrate than the barnacle (Tables III and IV). A half-day exposure to 0.55 mg. Cu/liter was sufficient to kill the three mussels in this concentration. One animal in each of the experiments succumbed following a half-day exposure to a concentration of only 0.14 mg. Cu/liter. With adult barnacles, exposures of two days and five days are required for killing at these concentrations. In these tests, and in many others with mussels, death did not ensue until several days after the animals had been transferred to fresh sea water. In one case the mussel did not die until the tenth day following a half-day exposure to the poison. In most cases the animals showed no outward sign of unhealthy condition in the interim.

TABLE III

Relation of killing time to concentration of cupric citrate in the mussel, Mytilus edulis. In each test three animals (about 3 cm. long) were placed in each toxic solution for the periods indicated and then transferred to fresh sea water. Started August 6, 1942

Concentration mg. Cu/liter	Time in toxic days	Killing time days
0.55	½	4, 8, 10
	1	2, 3, 4
	2	2, 3, 3
	3	2, 3, 3
0.14	½	4, *, *
	1	3, 3, 4
	2	3, 4, 4
	3	3, 4, 4

* Still alive when experiment terminated on August 18.

TABLE IV

Relation of killing time to concentration of cupric citrate in the mussel, Mytilus edulis. In each test two animals (about 3 cm. long) were placed in each toxic solution for the periods indicated and then transferred to fresh sea water. Started August 10, 1942

Concentration mg. Cu/liter	Time in toxic days	Killing time days
0.14	½	5, *
	1	3, 4
0.08	½	*, *
	1	*, *
	2	6, 8
0.04	½	*, *
	1	*, *
	2	*, *
	3	8, *
0.02	1	*, *
	2	*, *
	4	*, *

* Still alive when experiment terminated on August 20.

Accumulation of copper in barnacle tissues during poisoning and recovery

Several series of experiments were undertaken to determine the extent to which copper accumulated in the tissues of the barnacles subjected to solutions of cupric citrate. Since the shell material of the barnacle is several times greater in volume and many times greater in dry weight than the soft tissues, a technique was developed for the copper assay of both constituent parts of the animal separately. The number of barnacles used for a single analysis varied from 15 to 40 with the size and expected copper content. In the earlier experiments, the animals were chiseled free from the substratum, but in so doing all the fluid material was lost, and bits of broken shell were sometimes included with the soft parts. In the later experiments the soft tissues were taken from the shell without disengaging the animals from the substratum by removing the operculum and picking out the soft parts with forceps. The fluid material, which was removed with a pipette, and the tissues were then placed in a weighed Erlenmeyer flask. In the case of mussels, a much smaller number of animals was used. The relatively large amount of sea water within the mantle cavity of the mussel was drained off and discarded before removing the soft parts.

The wet weight of the soft tissues of barnacles and mussels was obtained and the approximate volume of the wet tissue was determined by forcing it into a graduated cylinder.[3] The material was transferred quantitatively to a flask, dried in an oven at 100° C., and the dry weight determined. Enough H_2SO_4 was added to char the material. It was heated to fumes on a hot plate, and concentrated HNO_3 was added dropwise until a colorless solution was obtained. The solution in the flask was transferred quantitatively to a 100 cc. volumetric flask and diluted to the mark. Test for copper was made by the carbamate method (Coulson, 1937). A blank to which the same amount of reagent was added was also carried through the above procedures. In the analysis of barnacle shells, the wet weight of the shells was first

[3] In the early experiments, volume in cubic centimeters alone was determined, but was subsequently found to be very nearly equal to the wet weight in grams.

determined. The shells were then dried in the oven at 100° C. overnight and the dry weight determined. The material was ignited in the muffle furnace at 800°–900° C. for three hours, removed and cooled. Two cubic centimeters of concentrated HCl were added, warming if necessary to dissolve.[4] The remainder of the analysis followed the same procedure as with the soft tissues.

Determinations revealed that the amount of copper in the shell tissue of both normal and poisoned barnacles was quite variable. There was also no consistent change in the copper content of the shells in the case of batches of poisoned barnacles with increasing amounts of copper in the soft tissues. Accordingly, in the later experiments the laborious and rather unsatisfactory copper assay of the shells was omitted.

The copper content of barnacles freshly brought into the laboratory is many times greater than an equal volume or an equal weight of sea water (Table V). Evidently under natural conditions, the barnacle tends to accumulate copper in its tissues to a considerable extent.

TABLE V

Copper content of freshly collected barnacles
Woods Hole sea water contains 0.00001 to 0.00003 mg./cc.

Species	Source	Number	Copper in soft parts			Copper in shells		
			mg.	mg./cc.*		mg.	mg./cc.	
Balanus eburneus	Cotuit, July 28	50	0.0017	0.0014		0.141	0.027	
	Cotuit, July 28	50	0.0027	0.0024		0.097	0.014	
			mg.	mg./g. Wet wt.	mg./g. Dry wt.	mg.	mg./g. Wet wt.	mg./g. Dry wt.
Balanus eburneus	Cotuit, Aug. 20	50	0.015	0.0038	0.008	0.007	0.0003	0.0004
	Cotuit, Aug. 20	40	0.010	0.0019	0.014	—	—	—
	Cotuit, Sept. 12	30	0.016	0.0011	0.016	—	—	—
Balanus balanoides	Woods Hole, July	100	0.029	0.029	0.104	0.069	0.0070	0.0075
	Buzzards Bay	50	0.023	0.027	0.068	0.063	0.0065	0.0067

* May be assumed equivalent to mg./g. wet weight.

In experiments designed to test whether barnacles could accumulate copper in their tissues, it was found that the amount of copper present increased rapidly for each day that the animals remained in the toxic solution. At the end of four days of continuous poisoning in the two concentrations used, all the animals were dead (Blocks A and C, Tables VI and VII). However, in those cases in which the barnacles were transferred to fresh sea water after one or two days, the animals were restored to what appeared to be a healthy condition.[5] Analysis of the tissues of the barnacles which had been replaced in fresh sea water showed that the animals had not only recovered their activity but also had eliminated a certain amount of copper. These barnacles still contained, however, as much as twenty-five times the amount of copper found in their tissues before poisoning began.

[4] If an excess of $CaCO_3$ remained, more acid was added until evolution of CO_2 ceased.

[5] The recovery of Bugula larvae from copper poisoning was reported by Miller and Cupp (1942) and Miller (1946).

TABLE VI

Changes in copper content of soft tissues during poisoning and recovery

Toxic: Cupric citrate, conc. 0.20 mg. Cu/liter
Test Animal: Balanus eburneus
Date: August 3–8, 1942
*Basis: Copper content per cubic centimeter of soft tissue **

Block A		Block B	
Initial copper content	0.010 mg.	Initial copper content	0.010 mg.
After 1 day in toxic	0.034 mg.	After 1 day in toxic	0.034 mg.
After 2 days in toxic	0.070 mg.	After 1 day in sea water	0.033 mg.
After 3 days in toxic	0.093 mg.	After 2 days in sea water	0.030 mg.
After 4 days in toxic	0.112 mg.	After 3 days in sea water	0.035 mg.
		After 4 days in sea water	0.026 mg.
Condition at end—dead		Condition at end—open, inactive but reacts to touch	

* May be assumed equivalent to wet weight.

TABLE VII

Changes in copper content of soft tissues after varying exposures to toxic and after recovery

Toxic: Cupric citrate, conc. 0.35 mg. Cu/liter
Test Animal: Balanus eburneus
Date: August 10–17, 1942
*Basis: Copper content per cubic centimeter of soft tissue **

Block C		Block D		Block E	
Initial Cu content	0.002 mg.	Initial Cu content	0.002 mg.	Initial Cu content	0.002 mg.
After 1 day in toxic	0.024 mg.	After 1 day in toxic	0.024 mg.	After 1 day in toxic	0.024 mg.
After 2 days in toxic	0.073 mg.	After 2 days in toxic	0.073 mg.	After 1 day in sea water	0.029 mg.
After 3 days in toxic	0.089 mg.	After 1 day in sea water	0.056 mg.		
After 4 days in toxic	0.075 mg.				
		After 9 days in sea water	0.054 mg.	After 6 days in sea water	0.022 mg.
Condition at end—dead.		Condition at end—cirri give incomplete sweep or full sweep but slow.		Condition at end—normal rapid sweep of cirri.	

* May be assumed equivalent to wet weight.

These indications that barnacles can take up relatively large quantities of copper without being killed and can eliminate the poison to a certain extent were confirmed by more elaborate experiments which were designed primarily to test the effects of the toxic and of different periods of exposure and of recovery (Tables VIII to XI).[6] These experiments demonstrated that barnacles took up copper

[6] In these experiments the dry weight of the soft tissues to be analyzed was determined and the copper content stated on the more accurate basis of milligrams of copper per gram of dry weight. The dry weight for the soft parts of the barnacle was found to be about 5 per cent to 20 per cent of the wet weight. Since this group of experiments was conducted during late summer and autumn, there exists the possibility that the results may not be strictly comparable with the earlier work. The barnacles tested were necessarily older and larger, and were no doubt in a different physiological condition. It was noted that the animals became less active as the season progressed, and by late autumn they tended to remain closed for long periods. This last fact alone would be expected to cause an important difference in the rate at which toxic was absorbed. In general, the animals tended to survive longer in high concentrations of toxic in the autumn than they had in the summer.

TABLE VIII

Comparison of changes in copper content of soft tissues during continuous exposure to low concentration of toxic and intermittent exposure to higher concentration

Toxic: Cupric citrate, Block F: 0.14 mg. Cu/l.; Block G: 0.22 mg. Cu/l.
Test Animal: Balanus eburneus
Date: August 24 to September 28, 1942
Basis: Copper content per gram of dry weight of soft tissue
Exposure times indicated for toxic are cumulative totals

Block F		Block G	
Initial copper content	0.014 mg.	Initial copper content	0.008 mg.
After 1 day in toxic	0.05 mg.	After 1 day in toxic	0.037 mg.
After 2 days in toxic	0.08 mg.	(Replaced in sea water for 1 day)	—
After 7 days in toxic	0.33 mg.	After 2 days in toxic	0.095 mg.
After 15 days in toxic	1.09 mg.	(Replaced in sea water for 1 day)	—
(Replaced in fresh sea water)		After 3 days in toxic	0.18 mg.
After 18 days in sea water	0.39 mg.	(Replaced in sea water for 6 days)	—
		After 4 days in toxic	0.23 mg.
			(0.19 mg.)*
		(Replaced in sea water for 7 days)	0.17 mg.
		After 7 days in toxic	0.48 mg.
		(Replaced in sea water for 16 days)	0.51 mg.
Condition at end—full but slow sweep of cirri		Condition at end—full but slow sweep of cirri	

* Batch of dead barnacles.

faster from the more concentrated toxic media. With the slower rate of absorption at the lower concentrations, the animals appeared to withstand higher amounts of copper in their tissues. Thus the highest copper content observed for living barnacles, namely, 1.09 mg. per g. dry weight, was found after fifteen days in 0.14 mg. Cu/liter cupric citrate (Table VIII, Block F). In cases in which large amounts of copper were absorbed the barnacles tended to live longer and to remain in better condition when periods in sea water were alternated with periods in the toxic medium.

In cases where the copper content was determined for the dead barnacles, which were found among the living individuals in increasing number as the experiment progressed, it was almost invariably true that the amount of copper present was less than that for the living specimens from the same block. This observation suggests that the entrance of copper and its fixation in the tissues of the barnacle is not simply a mechanical process of diffusion or adsorption but is a process which is influenced in some way by the metabolic reactions of the living organisms.

Due to the facts considered above, it is not possible to arrive at any definite value for an absolute amount of copper which must be absorbed by the barnacle to kill it, nor at any rate of absorption above which death will ensue. The results suggest that killing may not be due to the destruction of some vital substance or process by the direct action of the copper as much as to a general depressing action of the toxic on some function, such as the feeding reaction, to the extent that death eventually follows.

TABLE IX

Comparison of changes in copper content of soft tissues during continuous and intermittent exposures to high concentration of toxic and continuous exposure to lower concentration

Toxic: Block H—Cupric citrate 0.75 mg. Cu/l. Continuous
Block I—Cupric citrate 0.35 mg. Cu/l. Continuous
Block J—Cupric citrate 0.75 mg. Cu/l. Alternating daily with sea water
Test Animal: Balanus eburneus
Date: September 21 to October 14, 1942
Basis: Copper content per gram of dry weight of soft tissue

Block H		Block I	
Initial Cu content	0.016 mg.	Initial Cu content	0.016 mg.
After 2 days in toxic	0.17 mg.	After 3 days in toxic	0.13 mg.
After 4 days in toxic	0.30 mg.	After 6 days in toxic	0.25 mg.
After 6 days in toxic	0.38 mg.	After 9 days in toxic	0.32 mg.
After 7 days in toxic	(0.33 mg.)*	After 11 days in toxic	0.76 mg.
After 8 days in toxic	0.48 mg.	After 14 days in toxic	(0.39 mg.)*
After 11 days in toxic	(0.84 mg.)*	After 14 days in toxic	0.49 mg.
		After 18 days in toxic	(0.43 mg.)*
		After 18 days in toxic	0.60 mg.

Block J

Initial Cu content		0.016 mg.
After 2 days in toxic,	1 day in sea water	0.16 mg.
After 4 days in toxic,	3 days in sea water	0.13 mg.
After 6 days in toxic,	6 days in sea water	0.31 mg.
After 8 days in toxic,	7 days in sea water	0.42 mg.
After 8 days in toxic,	8 days in sea water	0.25 mg.
After 10 days in toxic,	9 days in sea water	0.48 mg.
After 12 days in toxic,	11 days in sea water	0.50 mg.
After 12 days in toxic,	11 days in sea water	(0.48 mg.)*

* Batch of dead barnacles.

TABLE X

Changes in copper content of soft tissues during recovery from various exposures to toxic

Toxic: Cupric citrate 0.75 mg. Cu/l.
Test Animal: B. eburneus
Date: October 21–November 12, 1942
Basis: Copper content per gram of dry weight of soft tissue

	Treatment	Copper content	Condition
Block L	After 3 days in toxic	0.38 mg.	++
	After 10 days in sea water	0.31 mg.	+
	After 14 days in sea water	0.21 mg.	+
Block M	After 6 days in toxic	0.38 mg.	+
	After 7 days in sea water	0.36 mg.	+
	After 14 days in sea water	0.40 mg.	++
Block N	After 9 days in toxic	0.58 mg.	0+
	After 9 days in toxic	(0.39 mg.)	dead individuals
	After 7 days in sea water	0.43 mg.	+
	After 14 days in sea water	0.39 mg.	++

No barnacles were found to have been killed by the absorption of less than 0.19 mg. of copper per g. of dry weight—an amount which is ten or more times the normal content of the tissues. In many cases animals succumbed after about 0.4 mg. per gram of dry weight had been taken up by the tissues. In no case did the barnacles absorb more than 1.09 mg. of copper per gram of dry weight. Barnacles which had accumulated copper from toxic solutions to the extent of 0.5 mg. to 1.09 mg./g. dry weight, in some cases revived when replaced in fresh sea water and were still alive two or three weeks later. At the end of the period, when they had regained normal, or nearly normal, activity, they contained 0.3 to 0.5 mg. of copper per g. of dry

TABLE XI

Changes in copper content of soft tissues during recovery from exposures at high concentrations of toxic

Toxic: Block O—Cupric citrate 0.90 mg. Cu/l.
Block Q—Cupric citrate 0.75 mg. Cu/l.
Test Animal: B. eburnesus
Date: October 21–November 21, 1942
Basis: Copper content per gram of dry weight of soft tissue

	Treatment	Copper content	Condition
Block O	After 8 days in toxic	0.52 mg.	0+
	After 8 days in toxic	(0.48 mg.)	dead individuals
	After 8 days in sea water	0.42 mg.	0+
Block Q	After 9 days in toxic	0.53 mg.	0+
	After 9 days in toxic	(0.35 mg.)	dead individuals
	After 8 days in sea water	0.40 mg.	+
	After 15 days in sea water	0.35 mg.	++

weight. In most instances the barnacles eliminated copper from their tissues, especially in cases where a high concentration had been accumulated. Although the copper which had been absorbed was never entirely eliminated, and although the process was slower than for accumulation, the experiments show that barnacles have some ability to rid their tissues of copper.

Accumulation of copper in mussel tissue during poisoning and recovery

To determine the amount of copper taken up by the tissues of the mussel when subjected to copper solutions, groups of 25 mussels were placed in various concentrations of cupric citrate and were later transferred to fresh sea water after increasing periods of time. The media were renewed every day. The copper content of 20 fresh animals from the same source was assayed at the beginning of the experiment and batches of three to five animals were withdrawn from the various groups for copper assay after the indicated number of days during poisoning and during recovery (Table XII).

The initial copper content of the mussel tissue was 0.0109 mg. per gram of dry weight, roughly the same as for the barnacle. Since the wet weight of the soft parts of the mussel was similarly found to be about ten times the dry weight, it is

clear that the fresh mussel also contains many times the concentration of copper present in the sea water.

After the first day in the toxic media, the mussels in the more concentrated solutions had taken up six to eight times their original amount of copper, but in the weaker solutions, there was no consistent increase in copper content until after an exposure of three days. The group of mussels (Group 4) in the weakest solution had absorbed only about two and one-half times their initial copper content after five days—or less than half the amount the Group 1 mussels had absorbed in one day in a solution five times as strong. In addition, the mussels were found to be able to rid their tissues of copper effectively. One day's sojourn in fresh sea water was suffi-

TABLE XII

Changes in copper content of soft tissues of mussel during poisoning and recovery

Toxic: Cupric citrate
Test Animal: Mytilus edulis, 25 animals (5 cm. long) in each group
Date: August 19 to September 5, 1942
Basis: Copper content in milligrams per gram of dry weight of soft tissue

Exposure to toxic solution began August 19. The line marks the time when mussels were transferred to fresh sea water

Group	Concentration of Cu citrate mg. Cu/l.	No. of days in toxic	Content on indicated date							
			Aug. 20	Aug. 21	Aug. 22	Aug. 23	Aug. 24	Aug. 25	Aug. 26	Sept. 5
1	0.120	1	0.066	0.0196	—	—	—	—*	—	—
2a	0.082	1	—	0.0135	—	—	0.017	—	—	0.0085
2b	0.082	2	0.084	0.031	0.023	—	—	—	—	0.0077
3	0.049	3	0.0076	—	0.024	0.018	—	—	0.017	0.0100
4	0.027	5	0.0135	—	0.021	—	0.025	0.029	—	0.0130
Control	0	0	0.0109							

* Condition on August 25: Group 1—all dead
Group 2a—9 dead
Group 2b—10 dead
Group 3—3 dead
Group 4—4 dead

No further deaths to September 5.

cient to reduce the amount of copper in the mussels from the strongest solutions by more than 70 per cent. In cases where longer exposures were employed (although in lower concentrations) more time seemed to be required for the elimination of the copper—perhaps because it had penetrated to deeper tissues. At the end of about two weeks in sea water, the copper content of the mussel tissue was back to normal—a fact quite in contrast to the situation with the barnacle.

In spite of the fact that after one day in sea water the copper content of the Group 1 mussels had been reduced at least to the amount reached by the mussels in the weaker solutions, all the animals in this group died within five days. Some irreparable damage had been done by the single day's exposure to the high concentration of the toxic. In the lower concentrations only three to four animals died

despite exposures to the toxic for three to five times as long. It is possible that the mussels in the lower concentrations could eliminate copper from vital tissues as fast as it tended to enter.

Although the values obtained in this single experiment are somewhat irregular, two important differences are indicated by the results as compared with the results obtained with barnacles. At the same concentrations, the exposures which are lethal to mussels are considerably shorter than for barnacles. Similarly the mussels succumbed after accumulating amounts of copper in their tissues which were much lower than was the case with barnacles.

Tests on the Poisoning of Cyprids

During January, 1943, experiments were undertaken at Miami Beach, Florida, on the poisoning of barnacles in the cyprid stage. Especial interest centered on these observations because the cyprid is the stage in which the animal first attaches itself to the substratum. It is the opinion of many that once a barnacle becomes attached, it can be killed only with great difficulty and that even if it is killed, the shell does not drop off but remains firmly attached. Actually, attachment takes place in two steps: first, the attachment of the antennae of the cyprid, following which metamorphosis is begun; and second, the attachment of the base of the barnacle after metamorphosis has been completed.

Cyprids were obtained by suspending glass microscope slides in Biscayne Bay overnight. The cyprids were presumed to be those of *Balanus improvisus*, which is the commonest adult barnacle in the neighborhood. The slides were brought into the laboratory where the following details in the development of the cyprids were observed.

The cyprids are originally attached by their antennae. About 5 or 6 hours after attachment, the animal undergoes metamorphosis in the course of which the cyprid shell is moulted and the animal emerges as a more or less round body still adhering to the substratum by the original attachment which now appears as the center of the convex base of the animal.

As development proceeds the base of the animal, which was at first freely movable, tends to flatten and to be pressed against the substratum. About 24 hours after metamorphosis the base is completely flattened with relatively sharp edges. A pulsation of the central parts of the animal has begun by this time, and the operculum may open, but no cirri are extended. The base is not caused to move by the activity of the animal, as before, but any part of it, except the original central point of attachment, is easily dislodged by pressure with a needle.

After another 24 hours, the cirri may be extended and filter actively, and the main area of the base begins to adhere to the substratum. At first the base merely gives the impression of being sticky; later it becomes attached with sufficient firmness so that it cannot be dislodged without tearing the tissue. Not until the third day or later does hard material (presumably calcium carbonate) begin to appear in the base.

The attachment of the cyprid by its antennae takes place quickly, and the original attaching structure remains in existence at least until calcification begins. The attachment of the base of the newly metamorphosed barnacle is a slow process which provides only weak adhesion at first but which finally supplies a permanent and

TABLE XIII

Effect of different concentrations of cupric citrate in preventing the metamorphosis of barnacle cyprids attached to glass slides

Species: probably Balanus improvisus

Concentration mg. Cu/liter	Number of cyprids exposed	Number of cyprids which metamorphosed
116.	6	2 (moult incomplete)
58.	6	3 (moult incomplete)
23.	4	4
9.7	3	3
4.9	2	2
0.93	11	11
0.47	9	9
0.23	5	5
0.12	9	9

firmly cemented calcareous structure. Poisoning of the barnacle could theoretically be accomplished either in the attached cyprid stage or as a newly metamorphosed animal before the calcification of the base.

The susceptibility of the cyprid to poisoning was investigated by placing cyprids attached to glass slides in solutions of cupric citrate (Table XIII) and of mercuric chloride (Table XIV). In the case of the copper solutions, the cyprids metamorphosed successfully in concentrations as great as 23 mg. Cu/1 and solutions as strong as 116 mg. Cu/1 were only partially inhibitory. Concentrations about 100 times greater than those ordinarily tolerated by adult barnacles, therefore, were not capable of killing the cyprids before they completed their metamorphosis.

In the case of the mercury solutions, although an inhibitory effect was observed at a lower concentration (16.6 mg. Hg/liter) than for copper, much higher concentrations of the toxic were required to prevent metamorphosis than would ordinarily kill adult barnacles.

Failure to kill the cyprid may be due to an unusually high resistance of the animal to poison in this stage or to the relative shortness of the period between attachment and metamorphosis, or both. From these results it is probable that the metamorphosis of the attached cyprid cannot be stopped by any concentration of toxic which could practically be obtained from a paint.

TABLE XIV

Effect of different concentration of mercuric chloride in preventing the metamorphosis of barnacle cyprids attached to glass slides

Species: probably Balanus improvisus

Toxic solution mg. Hg/liter	Number of cyprids exposed	Number which metamorphosed
83.	5	0
16.6	6	3 (moult incomplete)
3.3	6	6
0.66	9	9
0.17	7	7
0.09	10	10

The susceptibility to poisoning of the newly metamorphosed barnacle was tested in a similar fashion by placing slides bearing animals in this stage in solutions of cupric citrate (Table XV). Much lower concentrations of copper were required to kill the newly metamorphosed barnacle than was necessary to prevent the cyprids from carrying out their metamorphosis. The relation between killing time and concentration is roughly of the same order of magnitude as observed for adult barnacles at Woods Hole. There is some indication, however, that the newly metamorphosed animals are slightly more resistant, as concentrations of 0.23 mg. Cu/1 to 0.47 mg. Cu/1 were required to kill in five days in contrast to concentrations of only 0.14 to 0.30 mg. Cu/1 for the older animals.

Although recently metamorphosed barnacles survived for several days in concentrations of cupric citrate of 0.12 mg. Cu/1 or lower, their development was very materially retarded. In the stronger solutions the base remained in the rounded

TABLE XV

Relation of killing time to concentration of cupric citrate in barnacles which had just completed metamorphosis

Species: probably Balanus improvisus

Concentration mg. Cu/liter	Killing time days
9.7	1
4.9	1–2
0.97	3
0.93	3–4
0.47	5
0.23	5
0.20	7
0.12	10

condition, and in concentrations down to at least 0.20 mg. Cu/1 the base never became calcified nor rigidly cemented to the substratum, although it might become flattened and adhesive.

In a small number of tests reported subsequent to this investigation by Pyefinch and Mott (1944), free swimming cyprids of *Balanus balanoides* were killed in 24 hours by 0.5 to 1.0 p.p.m. copper from cupric sulphate. Metamorphosis was completed in concentrations up to 7 p.p.m. but after metamorphosis the barnacles were killed in 3 days by 0.5 p.p.m. Mercury from mercuric chloride was found to be more toxic than copper. These results agree very satisfactorily with ours.

At still lower concentrations of copper, in the present experiments, a complete change in the effect of the metal evidently comes about, for development was accelerated. Animals in the lowest concentration tested, namely 0.06 mg. Cu/1, reached the stage of active filtering with their cirri in less than 48 hours after metamorphosis, although the controls did not attain this stage until about 2 days later. It appears that a small amount of copper acted as a stimulant to development, and that larger amounts of copper retarded development even though they may be insufficient to kill the animals outright.

Discussion

Adult barnacles, and perhaps young stages also, can absorb ten to possibly one hundred times as much copper as their tissues normally contain without apparent injury. They can eliminate a certain amount of this material also. Therefore, when copper is used as a toxic in an antifouling paint, a substance is employed which can be taken up with impunity in relatively large quantities by the barnacle and to a lesser extent by the mussel. The animals can recover from the effects of this poison, to a certain extent at least, when again bathed by fresh sea water.

In some cases mussels which had received a lethal dose of poison remained in an apparent healthy condition for several days after being replaced in fresh sea water before dying. No such latent period was observed in the experiments with barnacles.

Possibly related to the facts just considered is the indication that the action of copper as a poison is not so much the direct destruction of some tissue or vital material in the barnacle as a general retardation of life processes. In the case of the adult barnacle, subjection to copper solutions causes a slowing and eventual cessation of the filtering activity of the cirri. In the case of the newly metamorphosed stages, not only is movement inhibited but development is seriously retarded as well. Much more satisfactory as a poison would be some substance which struck directly and irreversibly at some vital point in the animal and which could not be eliminated from the tissues. With such an ideal poison, barnacles which received only small doses, due to unavoidable dilution, would eventually be killed when a lethal amount of the material had accumulated. We have shown that with copper, sublethal doses may possibly stimulate development and certainly may allow subsequent recovery. Evidence that copper may stimulate the attachment of larvae has been reported by Prytherch (1934) for the oyster and by Miller (1946) for *Bugula*.

The relative vulnerability of the various steps in the attachment and growth of barnacles has been revealed by this investigation. The original idea that barnacles could be eliminated from a ship's hull only by preventing their initial attachment is partly right and partly wrong. As explained above, the attachment actually takes place in two steps. There is little promise of using poison successfully to prevent the first step, namely the attachment of the cyprid by its antennae, since this apparently takes place in a matter of minutes. However, we should not lose sight of the very important, but as yet unrealized, possibility of finding a substratum toward which the cyprid would display an avoiding reaction or to which the cement of the antennae would not adhere.[7]

Our tests have demonstrated the practical impossibility of preventing by poison the metamorphosis of the cyprid or of killing it during the process. This conclusion is substantiated by the results of Pyefinch and Mott (1944) and is in agreement with their statement: "Settlement and metamorphosis can take place on a paint in other respects anti-fouling though death of the barnacle occurs after metamorphosis." Similarly, at the other end of the life cycle, after the adult barnacle has attained any considerable size, it can be poisoned only with great difficulty for the reasons already discussed.

[7] In certain tests under laboratory conditions, Pyefinch and Mott (1944) found that cyprids failed to settle in concentrations of copper as low as 0.03 p.p.m.

There remains, however, the possibility of effectively preventing the second step in the attachment process, namely the permanent cementing of the base of the newly metamorphosed animal to the substratum. Although a long exposure to copper or a high concentration is necessary to kill the barnacle outright at this stage, moderate concentrations of poison tend to retard development and possibly to prevent the formation of the calcareous base, or the cement for this base, with the result that the young barnacle never succeeds in establishing a firm attachment, and hence is eventually displaced. The newly metamorphosed stage, therefore, appears to be the most vulnerable to copper poisoning.

The indication is, then, that copper paints act by preventing attachment but do so chiefly during the second step of the process through interference with the final cementing of the base of the metamorphosed barnacle to its substratum.

Summary

1. Direct tests were performed on the concentrations and exposures of a variety of metallic salts necessary to kill barnacles. The toxicities of mercury, cupric citrate, cupric tartrate, cupric salicylate, and cupric aminobenzoate were found to be slightly less than the toxicity of basic cupric carbonate. The toxicity of silver is about equal to that of basic cupric carbonate, but the toxicity of zinc is very much less.

2. The rate of killing of barnacles by cupric citrate is proportional to the concentration of the toxic over the range tested.

3. An extremely high concentration of copper or of mercury salts was necessary to prevent the metamorphosis of cyprids attached to glass plates. The results show the difficulty of preventing the *initial* attachment of cyprids, or their metamorphosis, by the use of copper paints.

4. Moderate concentrations of cupric citrate seriously retard the development of the newly metamorphosed barnacles and prevent the second step in attachment, namely, the formation of the cemented calcareous base.

5. Exposure of the newly metamorphosed barnacles to very low concentrations of cupric citrate accelerated development beyond that of the normal animals.

6. The soft tissues of adult barnacles normally contain a much higher concentration of copper than does sea water. When placed in solutions of cupric citrate, barnacles absorbed more than 100 times the normal copper content of the tissues. In no case were barnacles killed by the absorption of less than 0.19 mg. of copper per gram of dry weight—more than 10 times the normal content. In some cases barnacles which had absorbed 0.5 mg. to 1.09 mg./g. from toxic solutions revived when replaced in fresh sea water.

7. It was demonstrated that when replaced in fresh sea water, barnacles can eliminate from their tissues as much as two-thirds of the copper which has been absorbed from toxic solutions.

8. Mussels are more sensitive to poisoning by cupric citrate than barnacles. When exposed to copper solutions, mussels take up copper more rapidly than do barnacles, and when replaced in fresh sea water, they eliminate it from their tissues more rapidly and extensively. In many cases in which a considerable portion of the copper was eliminated, the mussels nevertheless succumbed subsequently.

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* ceased publication.

[ ] incomplete.

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